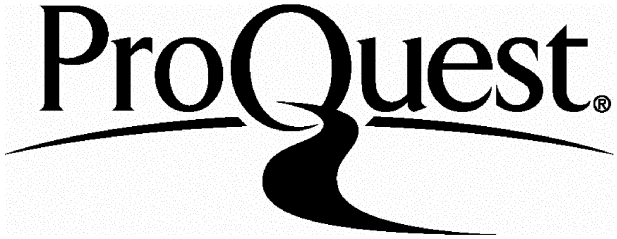
Investigation of Glucocorticoid and Local Immunological Function in Tuberculosis and Inflammation

by

Robert William Baker

A thesis submitted to the Faculty of Science of University College London, for the degree of Doctor of Philosophy

Department of Medical Microbiology
University College & Middlesex School of Medicine
The Windeyer Building
46 Cleveland St
W1P 6DB
Tel 0171 504 9155/ 9185
Fax 0171 504 8175
email robert.baker@ucl.ac.uk
2001



Dedicated to:

This thesis is dedicated to my wife Juliet and my children, Mattie and Ellie, without whom I might not have started but would probably have finished much sooner.

TABLE OF CONTENTS

1.1. Table of Figures

1.2. Table of tables

2. Acknowledgments

I would like to acknowledge the help, guidance and support of my supervisors, Dr Ali Zumla and Professor Graham Rook and my collaborators: Dr Brian R Walker, Professor Rory J Shaw, Dr John W Honour and Professor Stafford L Lightman. Support was provided by The Wellcome Trust, The Neuroendocrinology Charitable Trust, and the Middlesex Hospital Special Trustees. Technical assistance was provided by Dr. Ruth Best, Ms. Jill Smith and Mrs. Elvira Conway. BRW is a British Heart Foundation Senior Research Fellow.

3. Abbreviations

ACTH	Adrenocorticotrophic Hormone
AED	Androstenediol
BAL	Bronchoalveolar Lavage
CD	Clusters of differentiation
ConA	Concanavalin A
CRH	Corticotrophin releasing hormone
DC	Dendritic cells
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DTH	Delayed tissue hypersensitivity
GABA	Gamma-amino butyric acid
GC	Glucocorticoids
GCMS	Gas chromatography/ mass spectrometry
GMCSF	Granulocyte macrophage colony-stimulating factor
GR	Glucocorticoid receptors
GRE	Glucocorticoid response elements
HPAA	Hypothalamo-pituitary-adrenal axis
HSD	Hydroxysteroid dehydrogenase
IFN	Interferon
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LTB4	Leukotriene B4
MHC	Major Histocompatibility Complex

MR	Mineralocorticoid Receptor
mRNA	Messenger RNA
MV	Mycobacterium vaccae
NC	Nitrocellulose particles
Ova	Ovalbumin
PBMC	peripheral blood mononuclear cells
PMA	Phorbol Myristic Acetate
PPAR	peroxisome proliferator-activated receptor
PPD	Purified protein derivative
SHR	Spontaneously hypertensive rats
SLE	Systemic Lupus Erythematosus
TB	Tuberculosis
TCCD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGFβ	Tumour Growth Factor beta
Th-Cell	T(Thymocyte dependent) Helper Cell
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
TNF	Tumour Necrosis Factor

4. Summary

Tuberculosis is a unique disease in having been declared a global emergency by the World Health Organisation. Three million people are believed to die from infection with the bacillus each year. Understanding the pathogenesis of the disease is therefore a vital step towards developing new therapies and vaccines.

In this study the metabolism of certain crucial glucocorticoids - in particular cortisol and cortisone (corticosterone and dehydrocorticosterone in rodents) - has been analysed in the context of tuberculosis in humans and comparable inflammatory models in mice. Cortisol is an immunologically active glucocorticoid, where cortisone is its inactive form. Glucocorticoids, in their active form, influence the effectiveness of cell mediated immunity to disease. Conversely, products of cell-mediated immunity, particularly cytokines, influence the peripheral metabolism of cortisol. Cortisol metabolism has been addressed in health and disease, in humans and laboratory animals. The principal pathological state which has been investigated is tuberculosis.

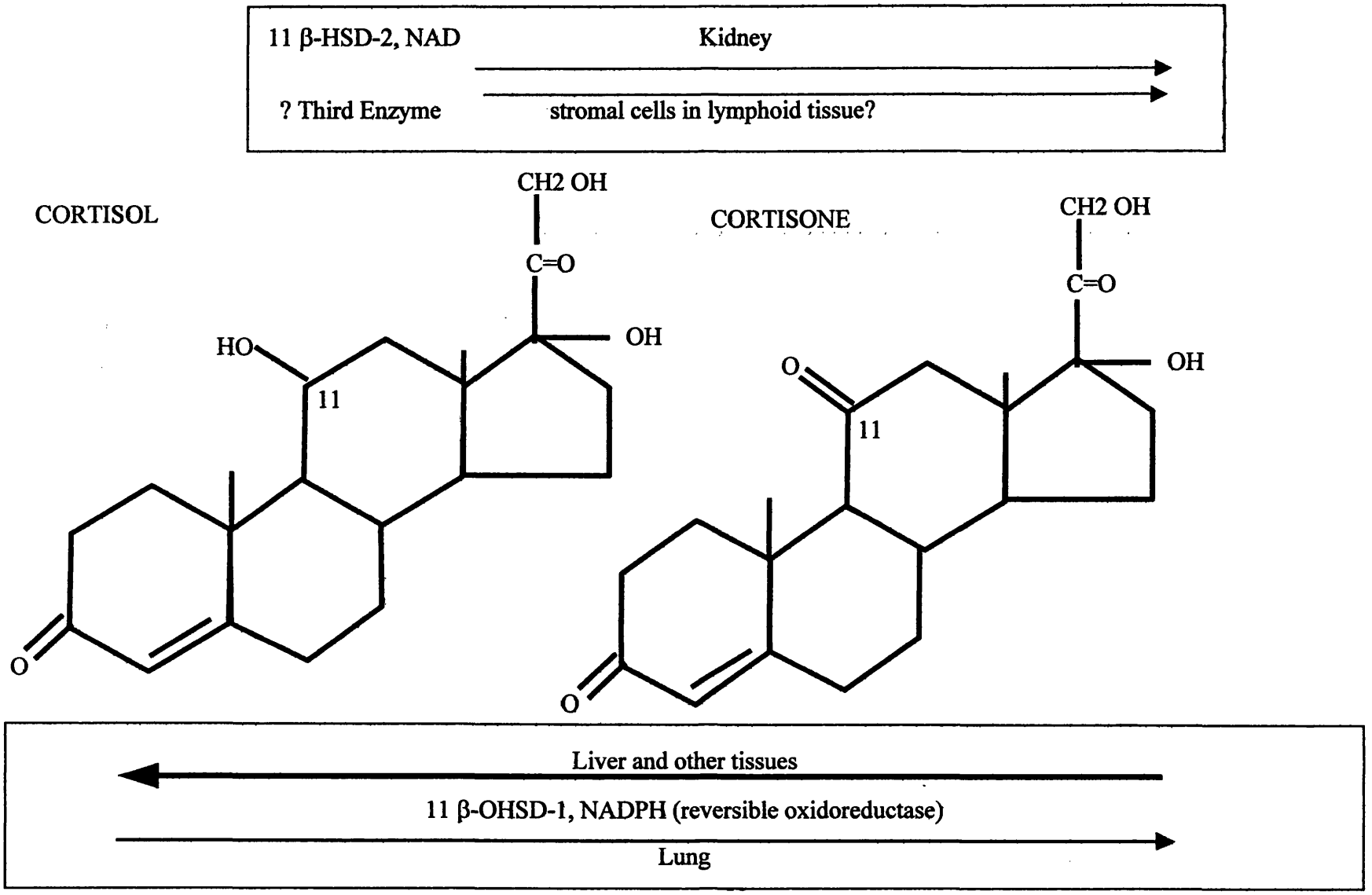
In the first part of the study, the central regulation of cortisol secretion via the hypothalamo-pituitary-adrenal axis was addressed in human tuberculosis, cured human tuberculosis and healthy humans. The HPAA was found to be intact in patients with pulmonary TB. Evidence of abnormal peripheral cortisol handling in acute human pulmonary TB was demonstrated, in that the conversion of inactive cortisone to its active and immunoregulatory metabolite cortisol is enhanced in patients with pulmonary TB. The enzymes responsible for this interconversion are the 11 beta-hydroxysteroid dehydrogenases.

In the second part of the study, the site of abnormal peripheral cortisol metabolism in humans with acute pulmonary TB was demonstrated to be within the lung. Based on the premise that the abnormality was likely to arise at the site of disease, and the observation that the enzyme 11-β HSD type 1 and 11-β HSD type 2 are present in lung, the interconversion of cortisol and cortisone at this site was analysed, and the ratio of cortisol to cortisone was found to be higher in the broncho-alveolar lavage of patients with TB than healthy volunteers or patients cured of TB. Analysis of cytokine production by lymphocytes in the lungs and peripheral blood was also performed, and although there was a trend for more CD4 and CD8 T-cells to produce Th2 cytokines, this was not found to be significant.

The study has taken the investigation of the hypothalamo-pituitary-adrenal axis further by assessing 24-hour urinary production of IL-6, an inflammatory cytokine involved with immunity to TB that is capable of stimulating the hypothalamo-pituitary-adrenal axis, and its soluble receptor S-IL-6R. No difference was found between IL-6 and S-IL-6R output in patients with TB, pneumonia or patients cured of TB when compared with healthy controls, an interesting observation given that overall cortisol output was not elevated.

In the third part of the study, activity of the enzyme 11-β HSD 1 in several different murine tissues was investigated in certain inflammatory models relevant to human disease. The findings confirmed that shifts in the equilibrium point of the cortisol-cortisone shuttle occur in sites of inflammation.

Figure 4-1 The cortisol-cortisone shuttle



5. General Introduction

A protective response to tuberculosis requires cell-mediated immunity, with macrophage activation and cytotoxic T cells, which are generated as a result of the activity of Th1 lymphocytes. Glucocorticoids can decrease the efficacy of Th1 lymphocyte-mediated immunity by direct impairment of the antimycobacterial effects of activated macrophages (Rook, Steele et al. 1987) and by causing newly recruited lymphocytes to deviate towards a Th2 cytokine profile (Ramirez, Fowell et al. 1996). This appears to be because cortisol reduces IL-12 from antigen presenting cells (APCs) and increases IL-10 (Vieira, Kalinski et al. 1998; Visser, van Boxel et al. 1998). Administration of synthetic glucocorticoids may induce reactivation of tuberculosis in man (Sahn and Lakshminarayan 1976; Haanaes and Bergmann 1983) and animals (Brown, Miles et al. 1995). Conditions of war, stress and social deprivation may lead to spontaneous reactivation of tuberculosis (Barr and Menzies 1994; Tocque, Regan et al. 1999) and it may be that elevated concentrations of the principal endogenous glucocorticoid, cortisol, are responsible. Moreover, tuberculosis is associated with release of IL-1, IL-6 and TNFα which drive cortisol secretion from the adrenal via activation of the hypothalamic-pituitary-adrenal axis (Besedovsky, del-Rey et al. 1991). However, studies of secretion and circulating concentrations of cortisol in patients with tuberculosis have yielded inconsistent data (Sarma, Chandra et al. 1990; Post, Soule et al. 1994). Rather than increased cortisol secretion in patients with tuberculosis, several reports suggest that the adrenal cortex is reduced in size, even in the absence of tuberculous infection of the adrenal, and responds less readily to exogenous adrenocorticotrophin (ACTH) administration. (Post, Soule et al. 1994; Reznek and Armstrong 1994; Hernandez Pando,

Orozco et al. 1995). Indeed, it has been suggested that inadequate secretion of cortisol in response to stress may account for the higher rates of sudden death in these patients (Onwubalili, Scott et al. 1986; Scott, Murphy et al. 1990).

Against this background a pilot study of urinary cortisol metabolites in patients with active pulmonary tuberculosis was performed (Rook, Honour et al. 1996). A reduction in the 24 hr output of glucocorticoid metabolites was noted in the most severely ill patients, but this was accompanied by a marked elevation in the ratio of metabolites of cortisol to those of its inactive derivative cortisone.

The interconversion of cortisol and cortisone is catalyzed by 11-β-hydroxysteroid dehydrogenases (11-β HSDs), which exist as at least two distinct enzymes (Figure 4-1) 11-β HSD type 2 is a high-affinity enzyme expressed in a number of sites, including blood vessels and the lung, and particularly the distal nephron, where it acts as an exclusive 11-dehydrogenase which converts cortisol to cortisone and thereby protects renal mineralocorticoid receptors from inappropriate activation by cortisol (Walker, Williams et al. 1994). In contrast, 11-β HSD type 1 is a low-affinity enzyme expressed in multiple tissues, including liver and lung (Berliner and Dougherty 1961; Schleimer 1991; Hubbard, Bickel et al. 1994). In most tissues, 11-β HSD type 1 acts as a reductase, converting cortisone to cortisol and increasing local glucocorticoid receptor activation. The elevated ratio of cortisol to cortisone metabolites in tuberculosis demonstrated in (Rook, Honour et al. 1996) could therefore reflect impaired 11-dehydrogenase activity and/or enhanced 11-reductase activity. Either way the result would be a net increase in tissue concentrations of cortisol. The potential importance of this factor is illustrated by the observation that in mice infected with *Listeria monocytogenes*, the inhibition of 11-β HSDs with glycyrrhetinic acid increases their susceptibility to infection in a similar manner to the potent synthetic glucocorticoid

dexamethasone (Hennebold, Mu et al. 1997). Abnormal peripheral metabolism of cortisol could also explain the discordant reports of reductions in total 24 hr cortisol output, and increases or decreases in responsiveness of the hypothalamo-pituitary-adrenal axis (Ellis and Tayoub 1986; Sarma, Chandra et al. 1990; Post, Soule et al. 1994; Rook, Honour et al. 1996). Impaired peripheral metabolism of cortisol will prolong its metabolic clearance rate, resulting in enhanced negative feedback at the hypothalamus and pituitary and a compensatory reduction in cortisol secretion to maintain normal circulating cortisol levels.

5.1. Cellular Immunity and Endocrinology

The distinction between endocrinology and immunology is an artificial one. T lymphocyte functions and cytokine profiles are constantly regulated by both systems. In particular glucocorticoid hormones (GC) and dehydroepiandrosterone sulphate (DHEAS) influence T lymphocytes. Sex steroids, among other steroid hormones, also affect T cells (mostly via stromal cells and antigen presenting cells or APCs) , but GC and DHEAS are the most important. They are present at similar concentrations in both sexes and have contrasting effects on immune function. GC are anti-inflammatory. They tend eventually to bias the lymphocyte response towards an interleukin-4 (IL-4) rich Th2 cytokine profile via APCs. This is inappropriate for the cell-mediated immunity required for resistance to intracellular parasites like *M. tuberculosis*.

The short term effects of GC are slightly more complex and are not discussed in detail here. Toxic chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD) can bind to steroid-type receptors affecting gene expression and causing immunosuppression similar to that resulting from GC.

DHEAS or one of its unidentified metabolites is, on the other hand, pro-inflammatory. It tends to oppose effects of GC and bias T lymphocyte secretion towards interleukin-2 (IL-2) and interferon gamma (IFNγ) and other Th1 cytokines. Th1/ Th2 ratios are crucial in murine and probably human infections. Increased susceptibility before infection with *M. tuberculosis* may be induced by immunisation that provokes a Th2 cytokine profile (Rook and Hernandez-Pando 1996).

Crude serum concentrations give a poor indication of the effects of GC and DHEAS. Target tissue response depends on diurnal rhythm of GC production, on regulation of metabolism and inactivation within the target organs themselves, and on the influence of other active compounds.

5.2. Glucocorticoids: Physiology

5.2.1. Cellular mechanisms of glucocorticoid activity

GC receptors (GR) are intracellular. Two isoforms - hGRα and hGRβ - of the human receptor may be yielded by alternative splicing of the primary transcript. They are bound to the 90 kDa heat shock protein (hsp90) and to other proteins of lower molecular weight . On binding to the receptor, the complex dissociates from hsp90 (Wilckens 1995). The resulting receptor-GC complex dimers can then interact with "glucocorticoid response elements" (GRE).These affect expression of numerous genes including some that encode cytokines and others that are involved in the regulation of inflammation and immunity (Figure 5-1)

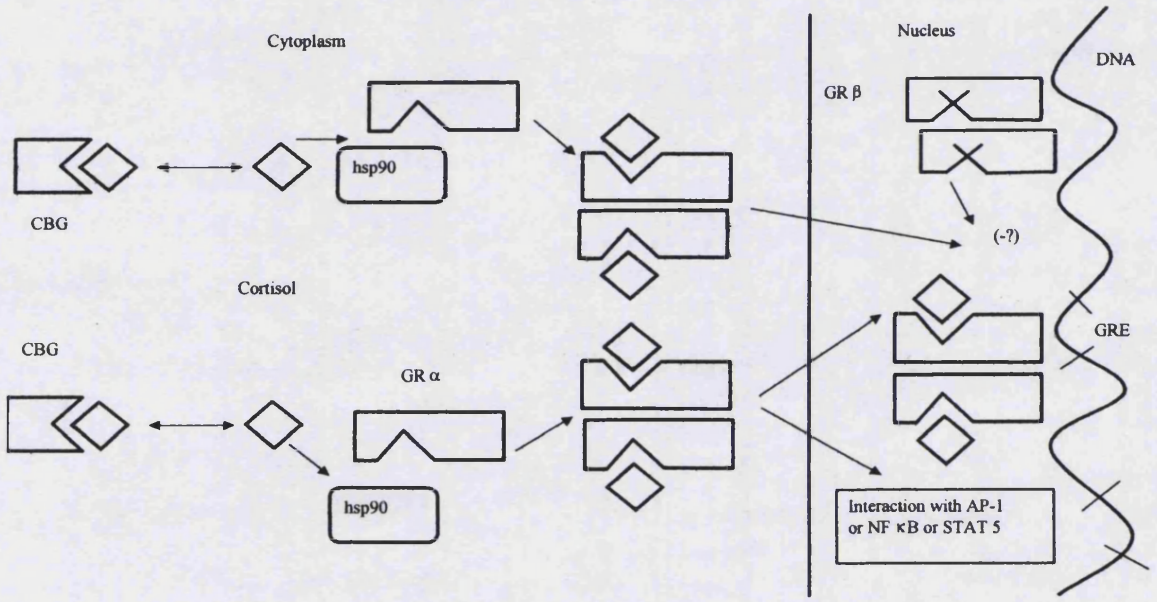


Figure 5-1 Schematic representation of cortisol in plasma

Cortisol in plasma is predominantly bound to corticosteroid binding globulin (CBG). Once free cortisol (rhomboids) has entered the cytoplasm it causes dissociation of the glucocorticoid receptor (GR or type 2 receptor) and mineralocorticoid receptor (MR or type 1 receptor) from the 90 kilodalton heat shock protein (hsp90) and other smaller proteins that are not shown. Receptor dimers are formed that travel to the nucleus and bind to glucocorticoid response elements that regulate transcription of the associated genes. MR dimers can act as functional antagonists of the GR, and an alternative transcript of the GR may also act as a physiological inhibitor [indicated by (-?)]. The receptor dimers can also interact with, and modulate the function of several other transcription factors such as AP-1, NFKB and STAT5 or perhaps the cortisol-GR complexes form heterodimers with such transcription factors.

The simple schematic representation in Figure 5-1 conceals many other complex interactions that make predicting the effects of GC at a cellular level very difficult. Some of these are outlined below.

5.2.2. GC-receptor complexes and other transcription factors

Other transcription factors such as AP-1, NFκB and JAK STAT are also influenced by receptor-glucocorticoid complexes (Wilckens 1995; Stöcklin, Vissler et al. 1996) These factors are also involved in regulation of gene transcription relevant to the immune response. Agonists that activate these pathways can profoundly modify or even reverse the effect of GC. This is of particular significance in the control of T cell apoptosis, discussed below.

5.2.3. Other glucocorticoid receptors

The β form of the GR mentioned above may function as a physiological inhibitor as it can inhibit the activation of gene expression by the α form (Figure 5-1) although its role is unproven (Oakley, Sar et al. 1996). The situation is complicated further by the existence of a second type of endogenous glucocorticoid receptor, known as Type 1, usually known as the mineralocorticoid receptor (MR). The GR already discussed is called Type 2. GC can bind to either type 1 or Type 2 (Figure 5-1) and in fact bind to MR with ten-fold greater affinity, with important consequences. MR as well as GR are both expressed in rat spleen. When exposed to ConA, a T lymphocyte mitogen, stimulation or inhibition of rat spleen T-cell proliferation depends on whether GC act via MR or GR. GC acting via GR inhibit proliferation, where it is

stimulating it via MR (Wiegers, Croiset et al. 1993; Wiegers, Reul et al. 1994). MR-GC complexes therefore functionally antagonise GR-GC complexes. This antagonism is further regulated by the diurnal rhythm of cortisol release (see below).

5.3. Other factors modulating glucocorticoid function in vivo

Measurement of circulating GC concentrations gives very limited information about the extent and nature of glucocorticoid-mediated effects *in vivo*. Effects depend on diurnal rhythms, and on metabolic regulation of GC action in peripheral tissues.

5.3.1. Diurnal rhythm of glucocorticoid secretion

In healthy individuals serum cortisol is secreted according to a diurnal rhythm. In man there is a series of peaks in the morning which decline throughout the day. Given the 10-fold higher affinity of cortisol for the MR than for the GR, this rhythm tends to result in occupation of both MR *and* GR in the morning, but only the MR later in the day. GR predominates almost exclusively in T cells in the thymus and peripheral blood. These lymphocyte GR mediate a mostly suppressive effect. T cells in the periphery and thymus therefore alternate between suppressed and non-suppressed states over a 24 hour period.

In an organ such as spleen that expresses MR as well as GR, the trough in the diurnal cycle of GC levels can result in antagonising effects mediated via the MR superimposed upon diminished GC effects. Rodents have an equivalent diurnal rhythm that is approximately the reverse of that seen in man, and have been shown to demonstrate this effect (Wiegers, Croiset

et al. 1993; Wiegers, Reul et al. 1994) The balance of these effects must vary naturally during the 24 hour cycle.

5.3.2. Lymphocyte function and the diurnal rhythm of cortisol

Diurnal GC-mediated cyclical variation in lymphocyte function may also occur in man. (Peterson, Waltenbaugh et al. 1992; Fukuda, Ichikawa et al. 1994; Petrovsky, McNair et al. 1994; Hiemke, Brunner et al. 1995). Plasma cortisol levels inversely correlate with both release of IFNγ in response to tetanus toxoid or PPD (Petrovsky, McNair et al. 1994) and the proliferative response of peripheral blood T cells to tetanus toxoid (Hiemke, Brunner et al. 1995). There is also cyclical variation in the response to oxazolone challenge in sensitised rats (Pownall, Kabler et al. 1979). Diurnal changes in circulating T cell subsets are comparable to those induced by prednisolone (Fukuda, Ichikawa et al. 1994).

These correlations may be due to coincidence, and not be relevant *in vivo*. Several other immunologically relevant diurnal rhythms exist that may be independent of the cortisol cycle. Interleukin-6 (IL-6) pursues a diurnal rhythm, with the highest levels throughout the night (peaking at 1.00 a.m.) with a nadir at 10.00 a.m. (Sothern, Roitman-Johnson et al. 1995). Complex and characteristic biphasic diurnal rhythms may be observed with tumour necrosis factor alpha (TNFα), interleukin-10 (IL-10) and granulocyte-macrophage colony-stimulating factor (GMCSF), while interleukin-2 (IL-2) displays a single mid-day peak (Young, Matthews et al. 1995). Soluble IL-2 receptors have a peak at 12.29 and a nadir at 4.14, so the peak is at least synchronous with IL-2 (Lemmer, Schwulera et al. 1992).

5.3.3. Disturbances of the diurnal rhythm of cortisol in disease.

The clinical and endocrinological convention has been to measure the early morning cortisol only, whereas from an immunological point of view that is the least revealing time point. A sample at 8.00 p.m. is much more enlightening. Abnormalities of the rhythm have been documented in specific situations which are also characterised by profound immunological disturbance system; chronic infection and stress.

Loss of the diurnal plasma cortisol rhythm has been described in some tuberculosis patients (Sarma, Chandra et al. 1990). This may occur in other chronic infections. ACTH and cortisol rhythms are blunted in some human immunodeficiency virus (HIV) seropositive subjects (Lortholary, Christeff et al. 1996). CD4+ cell numbers also demonstrate a daily rise between 0800 and 2200; this is blunted in HIV (Malone, Simms et al. 1990), or according to one report, ablated altogether (Martini, Muller et al. 1988). Cortisol rhythm is also lost during acute stress caused by strenuous physical exertion accompanied by sleep and energy deprivation (Opstad 1994).

5.4. The regulatory role of cortisol metabolism in the periphery

Peripheral tissues depend only partly for their GC regulation on concentration of cortisol in circulating plasma. Each is able to adjust its local steroid environment to suit its particular needs.

5.4.1. Glucocorticoid metabolism by peripheral tissue

GC-sensitivity of individual cells will be affected by receptor numbers, or affinity, or variations in the signalling pathways. Metabolism of cortisol itself may be another factor. It has long been known that some strains of fibroblasts are resistant to cortisol-mediated growth inhibition. These fibroblasts are able to catabolise cortisol via a variety of oxidations and reductions (Dougherty, Berliner et al. 1960; Berliner and Dougherty 1961). Individual lymphocytes may vary in their sensitivity to GC in health and disease. Lymphocytes from patients with systemic lupus erythematosus (SLE) are able to catabolise cortisol more effectively than normal. Lymphocytes from patients with rheumatoid arthritis do not share this property (Klein, Lishner et al. 1990).

5.4.2. Glucocorticoid metabolism - the cortisol-cortisone shuttle

The renal MR shows the same higher affinity for GC as elsewhere. The enzyme 11 β-hydroxysteroid type 2 (11-β HSD 2) converts cortisol into inactive cortisone (Figure 4-1) and so protects the MR from inappropriate binding to circulating cortisol (Walker 1994). Aldosterone is therefore the principal mediator of renal mineralocorticoid function although this hormone is present at much lower concentrations than GC. In normal individuals the liver oxidoreductase 11βHSD-1 then rapidly converts the resulting cortisone back to cortisol, so that the circulating cortisol:cortisone ratio tends to equilibrate at 5-10:1 (Figure 4-1).

Other organs such as lymph nodes and lung also possess 11-β HSD (Berliner and Dougherty 1961; Hubbard, Bickel et al. 1994; Daynes, Araneo et al. 1995; Rajan, Chapman et al. 1995) but they may not be either the conventional type 1 or type 2 enzymes. Lymph node 11-β HSD

may resemble a novel isoform recently observed in a choriocarcinoma line (Deckx and De Moor 1996; Gomez-Sanchez, Cox et al. 1996; Hennebold, Ryu et al. 1996) while the enzyme in the lung resembles the one in the liver (Rajan, Chapman et al. 1995) (Figure 4-1). The lung does, however, contain 11-β HSD 2 (Suzuki, Sasano et al. 1998) as do blood vessels (Bray, Du et al. 1999). In normal lungs the enzyme operates as a dehydrogenase, the reverse of the liver, converting cortisol into inactive cortisone (Hubbard, Bickel et al. 1994). The type 1 enzyme is clearly a reversible oxidoreductase. Removal of contaminating T cells from granulosa cell preparations alters the activity of the enzyme, implying that it may be regulated by cytokines (Evangelatou, Antoniw et al. 1996).

5.4.3. Cortisol metabolism in disease

Analysis of GC metabolites in urine has shown a significant switch in the cortisol/cortisone balance towards active cortisol in tuberculosis (Baker, Zumla et al. 1996; Rook, Honour et al. 1996, Baker, Walker et al. 2000). The site of this abnormality was not demonstrated, but a cytokine-mediated effect on the type 1 or type 2 enzymes in the lung is an obvious possibility (Hubbard, Bickel et al. 1994).

5.5. Glucocorticoid antagonism by dehydroepiandrosterone

Dehydroepiandrosterone sulphate (DHEAS) is the most copious steroid produced by the human adrenal after adrenarche. Adult adrenals secrete 10-15mg of DHEAS/day but serum levels then fall steadily with increasing age. In young adults concentrations are approximately 4μg/ml. Either DHEA itself, or unidentified metabolites clearly oppose some functions of GC.

(Blauer, Poth et al. 1991; Wright, Porter et al. 1992; Daynes, Araneo et al. 1995). DHEA is also a precursor for oestrogens and testosterone, and has several other poorly understood functions.

DHEAS is strongly bound to albumin and is reabsorbed by renal tubules. Eventually most undergoes conversion to DHEA which mostly circulates freely, although some weakly binds to albumin.

5.5.1. Differences in physiology of rodent DHEA

The metabolism of DHEA in rodents is clearly quite different from its physiology in man. Rat plasma contains <1ng/ml of DHEAS (Robel and Baulieu 1995) compared to about 4μg/ml of DHEAS in adult human plasma. Rodents tend to concentrate DHEAS in the brain - rat brains contained an average of 3.5 ng/gm of tissue over a 24 hour period (Robel and Baulieu 1995). DHEAS in brain has clear neurological functions that are not considered here. 17α-hydroxylase required to synthesise DHEAS is not present in rat adrenals. If the rodent adrenal does make DHEA it must use alternative pathways, as DHEAS and fatty acid esters of DHEA were found in the adrenals of rats at 5.1 ± 2.6 and 16.1 ± 6.4 ng/gm of tissue respectively (Robel and Baulieu 1995). It is possible that the immunological functions of DHEA are performed by another analogue or metabolite synthesised by a different pathway. DHEAS in brain is thought to be locally synthesised.

5.5.2. DHEA and rodent immune responses

It is therefore difficult to interpret the reported effects of DHEA or DHEAS in rodents. The literature demonstrates confusion about the appropriate dose of the steroid to employ in physiological studies of its effect on immune function. Doses of 1 gm/kg have been used (Morfin and Courchay 1994) in a species with physiological levels <1ng/ml of plasma or gram of tissue. Such doses are rapidly metabolised to androgens and oestrogens which have immunological effects of their own (reviewed in (Wilder 1995)). Similarly supraphysiological doses of DHEA (10mg DHEA/mouse, i.e. about 0.5 gm/kg) enhance influenza immunisation in elderly mice (Danenberg, Ben-Yehuda et al. 1995). 120mg/kg/day DHEA has been shown to partially restore rat spleen cell mitogenic responses to endotoxin and concanavalin A previously suppressed with dexamethasone , and *in vitro* IgG production. *Cryptosporidium parvum* colonisation of the gut was also reduced by DHEA in rats (Rasmussen, Martin et al. 1993). Three daily injections of 1.2 mg DHEA (i.e. 50-60 mg/kg/day) were found to block the ability of dexamethasone to render peripheral lymphocytes of mice unresponsive to mitogens, and prevent involution of the thymus (Blauer, Poth et al. 1991).

DHEA, using acceptable doses in the low μg range, appears to oppose GC, and to promote a Th1 cytokine pattern. It enhances production of Th1 cytokines such as IL-2 and interferon gamma (IFNγ) (Daynes and Araneo 1989; Daynes, Araneo et al. 1990; Daynes, Dowell et al. 1991; Daynes, Araneo et al. 1995). Optimal inhibition of GC-induced thymic involution in mice may be obtained with only 10-20μg/mouse of DHEA or of its derivative 3,17-androstenediol (i.e. 0.5 mg/kg; Al-Nakhli *et al.*, PhD thesis). This is 1% of the dose used by Blauer and colleagues (Blauer, Poth et al. 1991). Similarly, a single s.c. injection of 100μg DHEAS in propylene glycol, or 10μg DHEA dissolved in ethanol, given to aged mice 3 hrs before immunisation with pneumococcal polysaccharide produced higher antibody titres and

increased numbers of plaque-forming cells (Garg and Bondada 1993). DHEA has been shown to restore diminished immune functions in elderly mice, and to correct the dysregulated spontaneous cytokine release seen in old animals (Daynes, Araneo et al. 1993; Garg and Bondada 1993). These immunological effects are the exact converse of glucocorticoids which often enhance Th2 activity and synergise with Th2 cytokines (Fischer and Konig 1991; Wu, Sarfati et al. 1991; Guida, O'Hehir et al. 1994; Padgett, Sheridan et al. 1995).

5.5.3. DHEA and human immune responses

Properties of restoring decrepit immune function have been investigated in aged humans (Morales, Nolan et al. 1994). DHEA enhances IL-2 secretion from human peripheral blood T cells (Suzuki, Suzuki et al. 1991), and it has been shown that DHEA or 3β,17β-androstenediol (AED) will enhance mitogen-stimulated production of IFNγ from murine or human T cells in the range 10^{-7}-10^{-8}M (Al-Nakhli *et al.*, PhD thesis).

5.5.4. "Anti-glucocorticoid" actions of dehydroepiandrosterone

The mechanism of the anti-glucocorticoid effects of DHEA is unknown . It does not appear to competitively bind to the GC receptor. There is a single unconfirmed report of a specific DHEA-binding protein in T lymphocytes (Meikle, Dorchuck et al. 1992).

In the brain DHEAS clearly binds to several cell-membrane-associated receptors and it is an antagonist of $GABA_A$ (Robel and Baulieu 1995). In the periphery DHEAS very rapidly inhibits arachidonic acid-induced platelet aggregation (Jesse, Loesser et al. 1995), suggesting the possibility of receptors in cell membranes. The effect was seen at physiological concentrations in man but not in rodents.

There are known to be many "orphan" steroid receptors. It is possible that an as yet uncharacterised DHEA metabolite acts as a "anti-glucocorticoid" via one of these. If, unlike DHEA itself, this metabolite was not available for conversion to sex steroids, it would permit the regulation of inflammation and immunity by DHEA independently of sexual functions. 7-hydroxylated DHEA derivatives have been suggested as performing this function, but the supporting data *in vivo* and *in vitro* are not convincing (Morfin and Courchay 1994; Padgett and Loria 1994).

DHEAS acts as a peroxisome proliferator when given in high concentrations to rodents. This can only occur in the presence of peroxisome proliferator-activated receptor alpha (PPARα) (Peters, Yuan-Chun et al. 1996).

PPARα belongs to a family of transcription factors comparable to GC receptors. They bind to DNA motifs called PPAR-response elements and form heterodimers with RXR, the receptor for 9-*cis*-retinoic acid. This results in upregulation of expression of enzymes responsible for lipid homeostasis, fatty acid degradation, and destruction of leukotriene B4 (LTB4). Some authorities believe this effect could explain the restoration of immunological competence by DHEAS in elderly animals, via changes in cell membrane fluidity, phospholipid-dependent cell signalling pathways, and arachidonate-dependent mediators (Spencer, Poynter et al. 1995). This is an attractive theory, because there is a protein that binds DHEAS in liver, and PPARα is expressed at particularly high levels in liver and in the immune system (reviewed in Spencer, Poynter et al. 1995; Devchand, Keller et al. 1996). The theory is challenged by the fact that PPARα upregulates the enzymes that degrade LTB4 and this in turn limits inflammation (Devchand, Keller et al. 1996), whereas DHEA actually increases non-specific inflammation.

5.5.5. Dehydroepiandrosterone and the cortisol-cortisone shuttle

An attractive theory for the antiglucocorticoid action of DHEA is suggested by the influence of DHEAS upon the cortisol-cortisone shuttle, or its equivalent in rodents, the dehydrocorticosterone-corticosterone shuttle. The effect of DHEAS on 11-β HSD activity in spontaneously hypertensive rats (SHR) was investigated by Homma et al (Homma, Onodera et al. 1998), although this study suffered from the same flaw that the doses of hormone used were once again supraphysiological. SHR were given intraperitoneal injections of DHEAS (10 mg day-1 for 70 days) from six to 16 weeks of age. The dehydrocorticosterone/ corticosterone concentration ratio was significantly ($P < 0.05$) higher in the DHEAS group, suggesting that treatment with DHEAS enhanced the overall interconversion of corticosterone to dehydrocorticosterone. The activity of 11-β HSD in specific organs of the DHEAS group was affected, characteristic changes being increases in the kidney (14-58%), decreases in the liver (11-27%) and no change in the testis. Direct addition of DHEAS to 11-β HSD preparations from the kidneys of control SHR had the same effect as that observed in the in-vivo experiments. The fall in serum corticosterone in the DHEAS group was considered to be related, at least partly, to increased activity of kidney 11-β HSD. This would have the effect of diminishing circulating active corticosterone, and therefore be anti-glucocorticoid. These observations are pursued further below, using physiological concentrations of hormone.

5.5.6. Metabolism of DHEA in disease

Declining DHEA concentrations correlate with progression from HIV to AIDS (Wisniewski, Hilton et al. 1993). A deficit in DHEA relative to cortisol has been demonstrated in

tuberculosis (Rook, Honour et al. 1996). Both diseases involve a defect in Th1-mediated immunity.

5.6. T cell function and the stress response

A key feature of both psychological and physical stress is an increase in the production of cortisol, mediated by the hypothalamo-pituitary-adrenal axis. However not all of the immunological effects of stress are due to cortisol as other mechanisms, such as the adrenal medulla, are activated .

Few studies of the immunological effects of stress have addressed the issue of Th1/Th2 balance. Stress clearly does increase Th2 activity at the expense of Th1. Excessive exercise and deprivation of food and sleep results in raised cortisol, but no fall in DHEA, although its metabolite testosterone falls to castrate levels. The DHEA/GC ratio therefore falls and correlates with a fall in DTH responsiveness (Bernton, Hoover et al. 1995). Serum IgE levels, a marker of Th2 T-cell activity, also rise.

Chronic illness or after burn injury leads to marked decreases in DHEA concentrations (Araneo, Shelby et al. 1993), so DHEA/GC ratios can be more severely affected than in physiologically stressed but otherwise healthy individuals (Bernton, Hoover et al. 1995). In another study of exercise-induced and food/ sleep-deprived stress, Opstad found a rise in DHEAS accompanied by a fall in DHEA, showing that regulation of these hormones can be dissociated (Opstad 1992).

Stressed students about to sit examinations demonstrate evidence of classical switching from Th1toTh2. Epstein Barr virus is usually controlled by cytotoxic T cells and a type 1 response, and loss of control results in virus replication and increased antibody (Zwilling 1992). There is an increase in antibody to the virus in stressed students. mRNA for IFNγ and for the

glucocorticoid receptor is also diminished in the same circumstances. (Glaser, Lafuse et al. 1993).This effect appears to be mediated by APCs (Vieira, Kalinski et al. 1998; Visser, van Boxel et al. 1998).

The same effects can be demonstrated in controlled experiments on animals. Crowding or restraint stress can render tuberculous mice more prone to disease reactivation (Tobach and Bloch 1956; Brown, Sheridan et al. 1993). Even a small Th2 component affects this model (Rook and Hernandez-Pando 1996) .

5.7. Thymic T cell sensitivity to glucocorticoids

Radioresistant thymic epithelial cells contain steroidogenic enzymes. GC are therefore crucial regulators of T lymphocyte function (Vacchio, Papdopoulos et al. 1994). Such cells actively secrete steroids that may be central to thymic T lymphocyte repertoire selection (Vacchio, Papdopoulos et al. 1994). Immature thymocytes apoptose when exposed to GC, or the T cell receptor is engaged. These two signals, when both present, can antagonise each other, and within a critical range of balance both signals, apoptosis does not occur. The following hypothesis has been suggested by one group of researchers (Ashwell et al) . T cells incapable of binding MHC/peptide effectively, which are therefore immunologically weak, die from apoptosis signalled by GC alone. Where MHC/peptide T cell binding is intermediate the balance of the two signals protects from apoptosis. Potentially auto-reactive T cells that engage MHC/peptide with excessive avidity are eliminated because the antigen receptor signal for apoptosis is not adequately antagonised by the GC signal (King and Ashwell 1993; Vacchio, Papdopoulos et al. 1994).

In mouse thymocytes *in vitro* certain cytokines, particularly interleukin-4 (IL-4) and to a lesser extent IL-2 or IL-1 can protect from programmed cell death signalled by GC where the T cell receptor is not engaged (Migliorati, Pagliacci et al. 1992).

Thymic 11-β HSD, as previously mentioned (Dougherty, Berliner et al. 1960), must have some influence on these GC-mediated effects. It should be recognised that other non-GC steroid hormones also affect thymic function. The weight of the thymus increases after gonadectomy, while oestrogen and testosterone decrease it, possibly because thymic TGFβ expression is enhanced by testosterone (Olsen, Zhou et al. 1993). Sex steroids mediate these effects via stromal cells. Irrespective of these effects GC can, depending on the presence of other signals, and on the nature of the T cells, both enhance or inhibit programmed death of T cells.

5.8. In vitro *effects of GC on T cell activity*

Predictably the consequences for the immune response of exposure to GC are analogous to those caused by stress (Zwilling 1992; Brown, Sheridan et al. 1993; Bernton, Hoover et al. 1995), which are to polarise the T cell cytokine production towards Th2 (Fischer and Konig 1991; Wu, Sarfati et al. 1991; Guida, O'Hehir et al. 1994; Padgett, Sheridan et al. 1995). GC plus IL-4 have been shown to synergistically increase IgE production from human peripheral blood mononuclear cells (Bohle, Willheim et al. 1995). Experiments *in vitro* performed upon cultures of mixed T cell populations with GC are conflicting and difficult to interpret. The data are confounded partly by use of physiologically excessive GC concentrations , but more so by the delicate nature of regulation of T cell activity by GC and T cell receptors outlined

above. GC clearly affect specific T cell types in different ways, and according to which other signals are activated.

5.8.1. Effects of other agonists on GC on T cell function and apoptosis

GC do not act on T cells in an inert environment. The presence of other mediators makes interactions greatly more complex, as the following examples demonstrate. Dexamethasone and prostaglandin E2 act synergistically to inhibit both IL-2 secretion and subsequent proliferation of human T cells; this occurs in low concentrations that are not able to inhibit the responsiveness to stimulation by anti-CD3 (Elliott, Levay et al. 1996). Anti-CD28 was able to partly antagonise this suppression effect (Elliott, Brooks et al. 1992).

The complexity of interactions with GC in a complex biological environment is especially apparent when GC are considered as inducers of T cell apoptosis. CD44 ligands can suppress GC-induced apoptosis of T cells (Ayroldi, Cannarile et al. 1995). Peripheral T cells are often described as relatively insensitive to GC-induced apoptosis. This is not the case in the period immediately after mitogenic stimulation. Certain cytokines - IL-2,IL-4 and IL-10, in order of potency - protect peripheral T-cells from this effect, while puromycin and cycloheximide enhance it (Brunetti, Martelli et al. 1995).

5.8.2. Susceptibility of T cell subpopulations to GC effects *in vitro.*

T cells may be classified as CD4+CD45RO- (naive) and CD4+CD45RO+ (memory) subsets. These may be physically separated by fluorescence activated cell sorting (FACS). In one highly instructive experiment, these separated subsets were clonally expanded by priming with

anti-CD3 (solid phase) in the presence of IL-2 for 9 days. Clonally expanded subsets were then washed, put into fresh medium, and restimulated for 72 hrs with anti-CD3 and IL-2. Cultures were performed with and without addition of GC, both in priming and restimulation phases. Culture supernatants from the restimulation phase were then assayed for cytokines (Brinkman and Kristofic 1995). Addition of IL-2 to the experiment was essential because transcription of the IL-2 gene is downregulated by the GC receptor (Northrop, Crabtree et al. 1992), but the addition of that cytokine makes the data slightly difficult to interpret. Naive and memory cells nonetheless behaved quite differently, depending on which phase the GC were added to, in priming or restimulation. When cultured without steroids, both T cell types secreted cytokines in a Th0-like pattern, producing IL-5, IL-10, IFNγ, and in the case of the CD45RO+ memory cells, IL-4 as well. Naive cells cultured with GC tended to polarise towards Th2. They eventually secreted IL-10 if the GC was present during the priming phase, and both IL-4 and IL-10 if GC was present during the restimulation phase.

Memory cells, where GC was present during the priming phase, also switched to exclusive IL-10 production , but if GC was present during *restimulation*, they only produced IFNγ (Brinkman and Kristofic 1995).

This study reveals how confusion may arise when mixed cell populations are studied. It explains why misleading conclusions can be drawn from short term experiments with whole peripheral blood mononuclear cell populations stimulated with PHA. In such experiments IL-5 production is suppressed more easily than IL-2 or IFNγ (Van-Wauwe, Aerts et al. 1995). Similarly it explains why when T cell clones were grown from bronchoalveolar lavage (BAL) samples using PHA and IL-2 as the stimulus, dexamethasone (at supraphysiological doses) inhibited anti-CD3-induced production of IL-4 and IL-5 more than it inhibited production of IFNγ (Krouwels, van der Heijden et al. 1996) BAL T cells are known to be activated memory

cells, so this is compatible with the results of Brinkmann *et al.*, and does not alter the fact that if an immune response in naive cells is allowed to develop in the presence of GC, a Th2 line will develop. This has been rather clearly shown with spleen cells from "clean" laboratory rodents (Ramirez, Fowell et al. 1996), which have few memory cells under normal circumstances. This effect on T cells is believed to be mediated by APCs via the secretion of interleukin-10 (IL-10) and IL-12 (Vieira, Kalinski et al. 1998; Visser, van Boxel et al. 1998).

5.9. Overall effects of glucocorticoid action on T-cells

Even though GC readily inhibit secretion of Th2 cytokines from committed Th2 cells, their effect on naive cells is to bias their development towards Th2. Where Th1 cells are still able to secrete Th1 cytokines after GC exposure, their function is still blocked by other GC effects. These include increased TGFβ expression (Batuman, Ferrero et al. 1991), impaired macrophage function (Brown, Sheridan et al. 1993; Pownall, Kabler et al. 1979). Besides, *in vivo* in a dynamic situation what is important is the commitment of naive T cells towards Th2. Although the response to *M. tuberculosis* in human TB is dominated by Th1, thought to be the appropriate response for immunity, the response is clearly failing and peripheral blood mononuclear cells of patients secrete less IL-2 and IFNγ and more TGFβ and IL-10 than those of healthy contacts (Ellner 1997; Toossi and Ellner 1998). Increased expression of TGFβ and IL-10 are both driven by GC (Batuman, Ferrero et al. 1995; Vieira, Kalinski et al. 1998; Visser, van Boxel et al. 1998). Pulmonary tuberculosis is characterised by depression of purified protein derivative-stimulated (PPD-stimulated) blastogenesis in peripheral blood mononuclear cells (PBMCs) as well as decreased production of IL-2 and IFNγ. PPD directly stimulates the primed mononuclear cells from patients with TB to overproduce a panoply of cytokines including TGF-β and IL-10, which serve to depress PPD-stimulated blastogenesis

and cytokine expression. It is considered that the latter are downregulating cell-mediated immunity (Ellner 1997; Vanham, Toossi et al. 1997). All of these phenomena can be induced by raised cortisol levels, and GC effects may be at least partially achieved through modulation of the expression of the TGFβ 1 gene in activated T cells. For instance glucocorticoids cause increased production of TGFβ, a cytokine with potent T cell inhibiting activities, by human PBMNC. Dexamethasone causes an increase in TGF beta production and a dose- dependent two to fourfold increase in TGFβ 1 mRNA in activated as well as in unstimulated T cells. The increase in TGFβ 1 mRNA levels by dexamethasone is further potentiated two to threefold by cycloheximide, suggesting that the steroid effect may be due to inhibition of the synthesis of proteins that decrease TGFβ 1 gene transcription or the stability of its transcripts. In vitro nuclear transcription studies indicate the dexamethasone effects on TGFβ 1 gene expression to be largely transcriptional (Batuman, Ferrero et al. 1991). GC cause increased release IL-10 both *in vitro* (Visser, van Boxel et al. 1998) and, after major surgery at least, *in vivo* (Tabardel, Duchateau et al. 1996). Cortisol also downregulates the effectiveness of T-cells, macrophages and antigen-presenting cells. In endotoxin-stimulated dendritic cells (DC), GC strongly reduces the secretion of the Thl- skewing factor IL-12p70 (Vieira, Kalinski et al. 1998). Moreover as a result of their defective production of bioactive IL-12, GC-pretreated DC have a reduced ability to promote the production of IFNγ in CD4+ Th lymphocytes, confirmed by the observation that IFNγ production can be restored by exogenous IL-12. In contrast, GC treatment of DC enhances the secretion of the anti-inflammatory cytokine IL-10 and the type 2 cytokine IL-5 by the T cells (Vieira, Kalinski et al. 1998) This effect, in T-cells at least, is synergistic with IL-10. IL-10 and TGFβ 1 are able to potentiate the GC-induced inhibitory effect on T cell proliferation (Brunetti, Martelli et al. 1995).

Paradoxically conventional exogenous GC treatments for Th-2-mediated diseases such as eczema, asthma and hay fever may actually perpetuate the underlying problem by driving naive T cells towards Th2. Presumably synthetic GC exhibit anti-inflammatory effects by reducing cytokine production by Th2 cells (Corrigan, Hamid et al. 1995) and by increasing apoptosis of eosinophils (Meagher, Cousin et al. 1996).

Crucially in the management of TB it may be possible to exploit the anti-inflammatory effects of GC without simultaneously causing immunosuppression, or subjecting the immune system to an inappropriate Th1-to-Th2 switch.

5.10. Previous investigations of glucocorticoid function in tuberculosis

Clinicians have investigated adrenal function in human tuberculosis in the past with variable and inconclusive results. Post et al (Post, Soule et al. 1994) found that 9am cortisol levels were either within the normal range or marginally elevated in 50 patients, with adrenocorticotrophin (ACTH) levels undetectable in 32, normal in 17 and raised in one. All responded normally to synthetic ACTH. Sarma et al (Sarma, Chandra et al. 1990) reported raised basal cortisol levels in 27 newly diagnosed patients, 12 of whom had abnormally unresponsive synacthen tests. They also demonstrated loss of diurnal variation in cortisol secretion. Ellis and Tayoub (Ellis and Tayoub 1986) showed that 55% of 41 African Zulus with acute pulmonary tuberculosis had cortisol rises of less than 300nmol/l after 250μg of synacthen. DHEA Concentrations were also diminished in this study. Barnes et al (Barnes, Naraqi et al. 1989) showed normal or raised 9 am cortisols in 90 patients with acute tuberculosis, with subnormal ACTH responses in seven. These cortisol responses to synthetic ACTH fell within the normal range after treatment in all but one patient. The criteria for defining suboptimal synacthen responses were not uniformly applied in these studies. As explained below, this test as used in standard practice may be flawed.

It has long been known that human adrenals are enlarged in early tuberculous infection, and smaller later in the disease (Reznek and Armstrong 1994). This is confirmed by mouse models of tuberculosis, in which early Cushingoid hyperplasia of the adrenal is followed by profound atrophy (Hernandez Pando, Orozco et al. 1995). These mouse adrenals are not themselves infected. Sudden death in otherwise healthy humans shortly after commencing treatment for tuberculosis is a recognised phenomenon, and this may well be related to adrenal failure or cortisol reserve (Onwubalili, Scott et al. 1986; Scott, Murphy et al. 1990). Some studies have shown the incidence of this sudden unexplained death on commencing treatment to be as high as 1.6% (Ellis and Tayoub 1986).

Normal serum cortisol levels follow a diurnal rhythm, with a series of peaks in the morning followed by an evening trough. Studies that have assessed evening cortisols in tuberculosis have shown a remarkable loss of diurnal rhythm. The evening trough is absent (Sarma, Chandra et al. 1990). However hypo- or hyperadrenalism can be more sensitively and specifically assessed by other methods (Zumoff, Fukushima et al. 1974). Until recently the most sensitive method for picking up disturbances of adrenal steroid patterns - measurement and identification of steroid metabolites in 24 hour urine collections - had not been reported in detail in tuberculosis. Use of this technique has now revealed marked abnormalities in adrenal steroid output and metabolism (Rook, Honour et al. 1996) discussed below. Moreover, synacthen testing may not detect subtle abnormalities. Physiological concentrations of ACTH are in the low picogram levels, whereas the standard dose of synthetic ACTH used in stimulation tests is 250μg. Normal human adrenals will produce a maximal response with 500ng (Crowley, Holownia et al. 1991; Crowley, Hindmarsh et al. 1993) where 150ng will stimulate 70% of maximal output (Roberts, Barton et al. 1990). It is

therefore likely that the standard dose of synacthen will stimulate even an abnormally functioning adrenal. The response of the adrenal in tuberculous individuals clearly needed re-evaluation in the light of this information.

Studies of other adrenal metabolites in active tuberculosis have shown low levels of some androgens, in particular DHEA (Ellis and Tayoub 1986) both in serum and urine. The significance of this observation has only lately been addressed. The data were confirmed by a recent study (Rook, Honour et al. 1996) in which not only total output of adrenal androgens, but also total cortisol excretion - as measured by the sum of urinary metabolites (Zumoff, Fukushima et al. 1974) - were reduced in many patients by as much as 50%. However levels of tetrahydrocortisol - the metabolite most closely related to cortisol - were near normal. The reduction in total output of glucocorticoid derivatives was attributable to a drastic reduction in urinary derivatives of cortisone, an inactive metabolite of cortisol. A change in the balance of cortisol to cortisone in favour of active cortisol is compatible with the normal or elevated serum cortisol, and with a prolonged cortisol half-life, resulting in loss of the diurnal rhythm (Sarma, Chandra et al. 1990).

5.11. Conclusion

In this introduction I have attempted to point out the extreme complexity of the regulation of GC action on the T lymphocyte system, and how incomplete is the information derived from either measuring the 8.00 a.m. plasma cortisol level, or even more complete analysis of control of the hypothalamo-pituitary-adrenal axis via responses to synacthen or corticotrophin. GC function in TB clearly requires more complete analysis, and this is addressed in this thesis, based on the following hypothesis.

5.12. Hypothesis

The site of the previously demonstrated defect of glucocorticoid metabolism in tuberculosis, which leads to an excess of active cortisol at the expense of inactive cortisone, may be mediated centrally by the hypothalamo-pituitary adrenal axis, or peripherally via cytokine mediated activity on glucocorticoid regulating enzymes. This imbalance is immunomodulatory to the detriment of the host.

6. Recruitment of Human Subjects and Outline of Human Study Protocol

6.1. Patients and healthy controls

All human participants gave written informed consent for these studies which were approved by the local ethical committees of St. Mary's Hospital and University College Hospital, London. For all human participants, exclusion criteria included: corticosteroid or androgen therapy by any route during the preceding 12 months; abnormal thyroid or liver function tests; hypertension requiring medical therapy; diabetes mellitus; clinical depression during the preceding 12 months; obesity (body mass index >30 kg/m^2); previous autoimmune disease; positive HIV serology (all patients were tested for HIV infection).

Four groups of subjects were studied :

(i) patients with symptoms and chest Xray changes suggesive of active pulmonary tuberculosis confirmed by auramine staining of sputum or bronchoalveolar lavage fluid, and subsequent culture; the number of individuals confirmed by each method is shown in Table 6-1.

(ii) patients with previous pulmonary tuberculosis who completed treatment between 6 and 23 months before entering the study;

(iii) healthy volunteers with no clinical evidence of tuberculosis and on no medication.

(iv) a group of patients with a diagnosis of acute pneumonia, based upon clinical presentation, chest X-ray consolidation and/ or sputum culture

Individuals from groups (i), (ii) and (iii) participated in the first part of the study, a detailed investigation of the HPAA in pulmonary TB. Patients from group (iv) participated in a more limited study.

Individuals from groups (i) and (iii), participated in the second phase of the study - analysis of bronchoalveolar lavage.

Patients and healthy volunteers were matched for age, sex and ethnic origin (Table 6-1). Two patients cured of TB were recovering alcoholics with normal liver function. Among the patients with acute TB, there was one individual with angina, one with mild asthma (not on steroids), a crack cocaine addict with bacterial vaginosis and one patient with squamous carcinoma of the tongue. Five of the patients with active tuberculosis and three with cured tuberculosis withdrew from the study before completion of the protocol. Numbers are indicated for all measurements where results are discussed.

6.2. Human Studies of Endocrine Function in TB

The following measurements were made before any treatment: (i) 24-h urine collection for cortisol metabolites; (ii) intravenous cannulation at 0830 h following overnight fast and blood withdrawn after 30 min. supine at 0900 h for serum cortisol, renin activity, aldosterone, and electrolytes; and (iii) 4 hourly saliva collection (using Salivette, Sarstedt, Leicester, UK) for cortisol.

All healthy volunteers and patients with active or cured tuberculosis were then treated, once daily by mouth for all drugs, with pyrazinamide (<50kg body weight 1.5g, >50kg body weight 2g), pyridoxine (10mg), ethambutol (25mg/kg body weight) and isoniazid (300mg). Rifampicin, as an inducer of hepatic drug metabolising enzymes that reduces the

bioavailability of glucocorticoids (McAllister, Thompson et al. 1983), was omitted for the duration of the investigations. After 48 h, the following measurements were made on different days. Day 1: 24-h urine collection for cortisol metabolites and 4 hourly saliva collection for cortisol. Day 2: Corticotrophin releasing hormone (CRH) test in which 100 μg CRH (Ferring Pharmaceuticals Ltd. Feltham Middlesex UK) was injected at 0900 h and blood was withdrawn for 60 min from an intravenous cannula for cortisol and ACTH. Day 3: Overnight threshold dose dexamethasone suppression test in which 250 μg dexamethasone was taken orally at 2300 h, and subjects attended at 0900 h for cannulation and blood withdrawal for measurement of serum cortisol and dexamethasone. This was followed by administration of oral cortisone acetate (25 mg) and withdrawal of blood at intervals for 150 min for serum cortisol. Day 4: A second low dose suppression test performed as on day 3 but with 2 mg dexamethasone. This was followed by sequential stimulation with boluses of $ACTH_{1-24}$ (Synacthen, Ciba, Horsham, UK) 60ng, 150ng and 250μg at 90 min intervals) and withdrawal of blood at intervals for serum cortisol over 240 min.

Patients with pneumonia were studied during antibiotic treatment (Table 6-2). Samples were collected for baseline urine measurements as above. These patients were not given anti-tuberculous therapy, and did not participate in dynamic tests.

Table 6-1 Demographic details of human participants in study of endocrine function in TB

	Study 1: detailed assessment				*Study 2: bronchoalveolar lavage*	
	Healthy volunteers	*Acute tuberculosis*	*Cured tuberculosis*	*Acute pneumonia*	*Healthy volunteers*	*Acute tuberculosis*
Age (mean years (range))	30 (19-68)	39 (16-76)	48 (19-81)	30 (17-45)	30 (22-42)	37 (19-60)
Sex(Female/total)	6/13	8/17	6/14	1/6	2/11	5/13
Ethnic origin (Afrocaribbean/ Asian/ Caucasian)	4/4/5	4/4/9	2/2/10	0/1/5	4/1/6	3/2/6
Body mass index (kg/m^2)	23.02±0.46	20.9±0.5	20.1±0.5	not available	23.4±0.64	20.8±0.84
Hospitalised during study	0	11	0	5	0	9
Withdrawals during study	3	5	4	0	0	0
Tuberculosis confirmation (ZN staining/ culture)		17/12				13/8

Table 6-2 Details of patients with pneumonia

Sex	Ethnic Origin	Age	Organism	Treatment at time of 24hr Urine Collection	THF/THE ratio
m	Indian	34	Haemophilus influenzae	Clarythromycin/ Oxytetracycline/ Amoxycillin	0.96
f	Indian	17	Haemophilus influenzae	Nil*	0.58
m	Afro-Caribbean	30	Streptococcus pneumoniae	Clarythromycin/ Benzylpenicillin	0.5
m	Caucasian	45	?	Amoxycillin/ Erythromycin	0.77
m	Caucasian	16	?	Amoxycillin/ Erythromycin	0.98
m	Caucasian	37	Klebsiella pneumoniae	Clarythromycin/ Ceftazidine	1.59

* Collections made before treatment and during initial treatment for suspected, and subsequently excluded, TB. Ratio of urinary metabolites of cortisol and cortisone unchanged on treatment.

6.3. Statistical analysis

Variables were compared between groups, and across time-courses, by analysis of variance (ANOVA) followed by least squares difference tests where appropriate. Relationships between continuous variables were compared by multiple regression analysis. Non-parametric data were analysed by Mann Whitney U-test.

6.4. Methodology

A more detailed methodology for analysis of specific parameters is described in the text where appropriate.

Serum (Harbuz, Rees et al. 1992) and salivary (Harbuz, Rees et al. 1992) cortisol and serum ACTH (Cunnah, Jessop et al. 1987) were measured by radioimmunoassays, as were plasma renin activity and aldosterone (St. Mary's Hospital, Paddington, London, modifications of Menard and Catt 1972; Few, Chaudry et al. 1984). Serum dexamethasone was measured by gas chromatography and mass spectrometry (Best, Nelson et al. 1997). Cortisol binding globulin was measured by radioimmunoassay (Megdenix radioimmunoassay kit, Lifescreen, UK). Bronchoalveolar lavage cortisol and cortisone were measured by radioimmunoassay after separation by HPLC.

7. Abnormalities of glucocorticoid and androgen metabolites in 24 hour urine collections in pulmonary TB

7.1.1. Introduction

The reasons why cortisol metabolism in pulmonary tuberculosis warrants investigation are outlined in the introduction. Abnormalities of glucocorticoid and androgen metabolism in 24 hour urine collection in patients with TB have previously been described (Rook, Honour et al. 1996). In that study, controlled collection of 24-hour urine specimens was not performed. No controls with other infective pulmonary diseases were included, nor was an attempt made to establish whether there may be a constitutive defect in cortisol metabolism. The effects of drugs and hospitalisation were not adequately controlled for.

7.1.2. Methods

Patients with acute TB, healthy controls and patients with acute pneumonia were recruited as outlined above (chapter 6). Patients with pneumonia were studied during antibiotic treatment (Table 6-2). Samples were collected for baseline urine measurements as above. These patients were not given anti-tuberculous therapy, and did not participate in further tests. Urine was analysed for conjugated and unconjugated metabolites of cortisol and androgens by gas chromatography and mass spectrometry (GC/MS), as previously described (Rook, Honour et al. 1996).

7.1.3. Results

In patients with active pulmonary tuberculosis compared with other groups there was no change in overall cortisol production rate (Table 7-1) However there was a change in the metabolism of cortisol. The urines from patients with active pulmonary tuberculosis showed an increase in the ratio of metabolites of cortisol relative to metabolites of cortisone (Figure 7-1). The increase in ratio of cortisol to cortisone metabolites in patients with acute TB remained significant during 3 days of treatment in hospital. There was also an increase in the ratio of 5β- to 5α-tetrahydrocortisol in the patients with active pulmonary tuberculosis (Table 7-1Those patients with acute non-tuberculous pneumonia, who were on various antibiotic regimens at the time of sampling, showed a wide range in the ratio of cortisol to cortisone metabolites (Table 6-2). Gender, ethnic origin, body mass index and age did not significantly affect differences in ratio between groups.

In contrast to the previous study showing a reduction in metabolites of DHEA (Rook, Honour et al. 1996) in 24 hour urines of patients with pulmonary TB compared to healthy controls, this study did not demonstrate such an abnormality. There was a significant reduction in production of metabolites of DHEA in patients previously cured of TB (Table 7-2). However this reduction in production of DHEA metabolites correlated very strongly with age ($R=-0.38$, $p<0.005$). Patients cured of TB tended to be older (Table 6-1). Reduction in production of DHEA is a recognised function of aging (Morales et al, 1994). J Clin Endocrinol Metab **78**: 1360-1367.As there was no difference between patients with TB, healthy controls and patients with acute pneumonia, this was not thought to be significant.

Table 7-1Biochemical results from investigation of patients with TB and controls

	Acute Tuberculosis		**Cured Tuberculosis**		**Healthy Volunteers**		**Pneumonia**
Before/ during treatment	Before	During	Before	During	Before	During	During
Total cortisol metabolites excretion (μg/day)	4530±537	6459±857	3720±881	4563±1144	4779±564	5006±871	4182±732
(THF + alloTHF)/THE [1]	1.19±0.1	1.37±0.18	0.89±0.05	1.03±0.18	0.78±0.04	0.84±0.07	0.97±0.16
THF/alloTHF [2]	2.94±0.4	2.82±0.5	2.28±0.3	1.63±0.23	1.54±0.22	1.67±0.23	1.93±0.25
serum dexamethasone (ng/ml) after dexamethasone 250 mg		2.15±0.4		2.3±0.3		3.1±0.79	
serum cortisol (nmol/l) after dexamethasone 250 mg		271±63		174±40		139±44	
serum dexamethasone after 2mg (ng/ml)		8.54±1.9		9.92±1.59		7.0±1.1	
serum cortisol (nmol/l) after dexamethasone 2mg		47±8.6		48±6.5		38±3.9	
plasma aldosterone (pmol/l)	318±51.3		239±36		313±39.8		
plasma renin activity (ng/ml/h)	1.05±0.13		0.97±0.2		0.96±0.19		
Amplitude of diurnal variation in salivary cortisol from 8am-8pm (nmol/l)	0.43±0.93	1.12±1.1	3.65±0.91	1.67±1.03	3.75±1.2	3.28±0.9	0.95±1.27
8 p.m. salivary cortisol	2.27±1.12	1.93±1.19	0.69±0.27	2.02±1.3	0.49±0.08	1.06±0.35	3.12±1.27
serum K+ (mmol/l)	4.0±0.07		3.9±0.09		3.83±0.1		
Plasma cortisol binding globulin (mg/ml)	22.77±1.83		20.84±4.93		19.97±2.32		

1. **ANOVA** Before treatment Acute TB: Healthy: Cured TB $p<0.005$; Individual comparisons: Acute TB: Healthy $p<0.005$; Acute TB: Cured TB $p<0.01$. On treatment Acute TB: HV: Cured TB: Pneumonia $p<0.05$; Individual comparisons: Acute TB: HV $p<0.005$; Acute TB: Cured TB $p<0.01$; Acute TB: pneumonia $p<0.05$.

2 **ANOVA** Before treatment Acute TB: HV: Cured TB $p<0.05$; Individual comparisons: Acute TB: HV $p<0.01$.

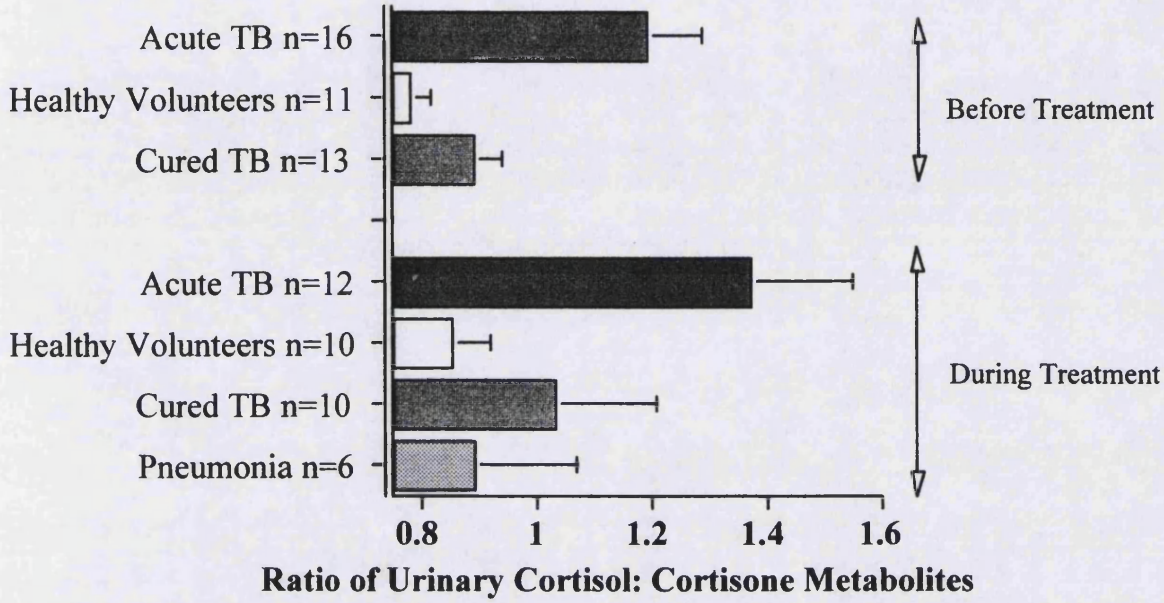


Figure 7-1 Ratio of metabolites of cortisol and cortisone in 24 hour urine.

Metabolites of cortisol (THF + alloTHF) to metabolites of cortisone (THE) in groups before (above) and during treatment (below), mean ± SEM, expressed as ratio =/- SEM. "During treatment" refers to antituberculous chemotherapy after three days in all groups apart from those with acute pneumonia, who were treated with various antibiotics and for varying durations. Patients with tuberculosis (TB) had significantly elevated ratios compared to healthy volunteers, cured patients or patients with pneumonia. ANOVA significant between groups before treatment $p<0.005$, significant between acute tuberculosis and healthy volunteers to $p<0.01$; significant between acute tuberculosis and cured tuberculosis $p<0.01$. On treatment ANOVA between groups $p<0.05$; significant between acute tuberculosis and healthy volunteers $p<0.005$; significant between acute tuberculosis and cured tuberculosis $p<0.01$; significant between acute tuberculosis and pneumonia $p<0.05$. See Table 7-1 for values.

Discrepancies in sample numbers compared to Table 6-1 relates to incomplete 24h urine collections.

Table 7-2 Results of analysis of DHEA metabolites in patients with TB and controls, μg/ day.

Acute tuberculosis		**Healthy volunteers**		**Cured tuberculosis**		**Pneumonia**	
Before	During	Before	During	Before	During	Before	During
2213±312	2876±393	2391±248	2751±402	1455±262	1465±171		2603±683

7.1.4. Discussion

These data confirm the earlier observation (Rook, Honour et al. 1996) that there is an abnormality of cortisol metabolism in patients with pulmonary tuberculosis. Further, the fact that the abnormality returns to normal three months after cure suggests that this is not a constitutive defect. The fact that the abnormality was present before and did not correct during treatment suggests that it is not due to drugs, hospitalisation or stress. The patients with acute pneumonia also failed to show the abnormality, suggesting that this phenomenon may not occur during all types of infection. Acute pneumonia and tuberculosis are different in terms of chronicity and inflammatory processes. It is possible that other pulmonary conditions, or other chronic conditions, might show the same abnormality.

8. Interconversion of cortisol and cortisone in pulmonary TB

8.1.1. Introduction

To investigate further the abnormality of the ratio of cortisol to cortisone in the 24-hour urine collections of patients with pulmonary TB, assessment of the accumulation of cortisol in peripheral serum following oral administration of cortisone was performed.

8.1.2. Methods

Patients with acute TB, patients cured of TB and matched healthy controls (as above) were administered 25mg cortisone acetate po stat at time 0. Dexamethasone 250 μg had been administered at 23.00 on the preceding evening for assessment of low-dose suppression. Dexamethasone is a synthetic glucocorticoid which suppresses endogenous secretion of cortisol by the adrenal, but is not detected in cortisol assays. Collections of serum were made from supine, resting subjects at t=0, 30, 60, 120 and 150 minutes. Serum cortisol was measured by radioimmunoassay (Harbuz, Rees et al. 1992).

8.1.3. Results

After similar suppression of plasma cortisol with 250 μg dexamethasone, and administration of 25 mg oral cortisone, peak plasma cortisol concentrations were higher in patients with active tuberculosis patients relative to other groups (Figure 8-1). Note that there were no differences in corticosteroid binding globulin (Table 7-1) or baseline plasma cortisol (Figure 8-1) between groups.

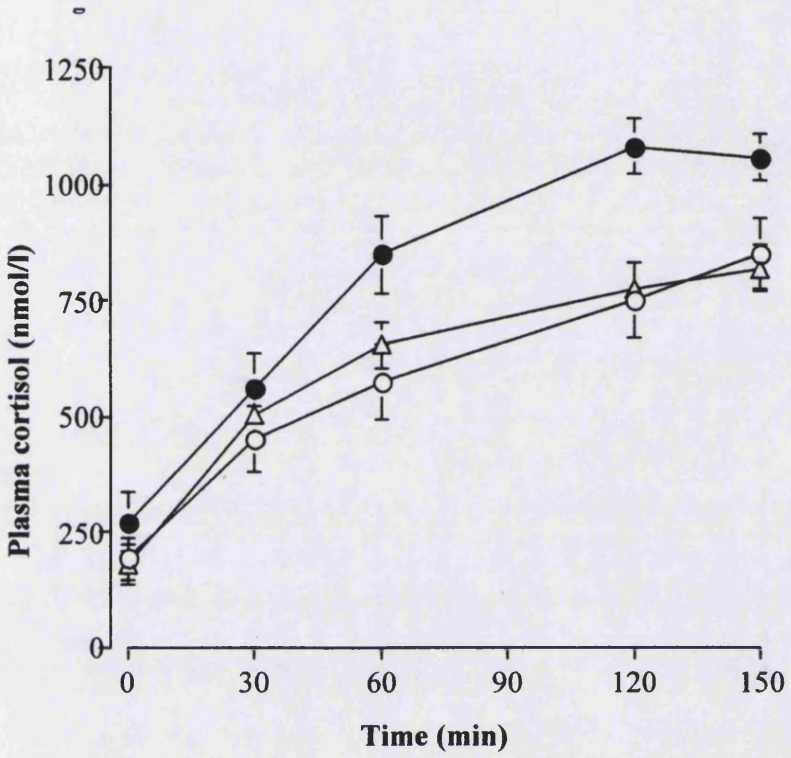


Figure 8-1 Accumulation of cortisol in serum following oral administration of cortisone acetate 25 mg and overnight dexamethasone 250 μg, mean ± SEM.

●: Patients with tuberculosis, n=14; Δ: Patients cured of tuberculosis, n=10; O: Healthy volunteers, n=10. Patients with tuberculosis achieved a significantly higher peak of plasma cortisol (1157±55 nmol/l) than healthy volunteers (882±73 nmol/l, p<0.005)

8.1.4. Discussion

This study does not address whether increased activity of 11-β HSD 1 occurs in all tissues where it is expressed, or only in some sites. The generation of cortisol in the peripheral circulation following oral cortisone administration is thought to reflect first pass metabolism in the liver (Walker, Campbell et al. 1992), but may also be influenced by cortisol/cortisone

equilibrium in other sites. The patients in whom this abnormality was detected had evidence of pulmonary disease. As has been described, 11-β HSD 1 occurs in the lungs. It would therefore seem a reasonable hypothesis that the abnormality of glucocorticoid metabolism arises at the site of disease. This is investigated further in chapter 10.

9. The hypothalamo-pituitary-adrenal axis in pulmonary TB.

9.1.1. Introduction

The hypothalamo-pituitary-adrenal axis governs central control of regulation of glucocorticoid secretion in health and disease. The end-point of the axis is secretion of cortisol by the adrenal gland; this is stimulated directly by ACTH produced in the pituitary which is in turn under the influence of CRH; negative feedback operates at each level. Synthetic glucocorticoids such as dexamethasone will suppress secretion of cortisol by a normal adrenal, but will not be detected by radioimmunoassay for cortisol. HPAA function may therefore be determined by a number of indices –

a) 24 hour urinary output of cortisol;

b) diurnal rhythm of cortisol secretion;

c) response of the adrenal to ACTH;

d) responses of the pituitary and adrenal to CRH

e) Suppression of adrenal output of cortisol by dexamethasone

f) target tissue sensitivity to glucocorticoids: Intact renal metabolism of cortisol in renal tubules by 11-β HSD 2 may also be inferred by plasma concentrations of aldosterone, renin and plasma renin activity.

9.1.2. 24 hour urinary output of cortisol

A key indicator of function of the hypothalamo-pituitary-adrenal axis is overall cortisol production rate. This may be estimated from the sum of the daily excretion of the principal urinary metabolites of cortisol and cortisone (5-tetrahydrocortisol (THF), 5α-tetrahydrocortisol (allo-THF), tetrahydrocortisone (THE), α- and β-cortols, and α- and β-cortolones) (Zumoff, Fukushima et al. 1974)

9.1.3. Methods

Patients were recruited as previously described; 24 hr urine collections were as for metabolites of cortisol and cortisone; the metabolites of cortisol and cortisone were determined by GCMS (Zumoff, Fukushima et al. 1974).

9.1.4. Results

In patients with active pulmonary tuberculosis compared with other groups there was no change in overall cortisol production rate, as measured by GCMS in 24-hour urine collections. (Table 7-1). There was no significant change in cortisol production rate after three days of treatment.

9.1.5. Discussion

Normal cortisol production rate is prima facie evidence of intact adrenal function in individuals with acute tuberculosis, cured TB and healthy controls. It is of particular note that cortisol production was not increased. These data do not address central regulation of cortisol secretion by the HPAA. They do, however, refute the suggestion that the abnormalities

described are a non-specific response of the adrenal to the "stress" of chronic disease. The adrenal is sensitive to neuroendocrine as well as immunological stimuli, including inflammatory cytokines; clearly other aspects of central control of cortisol secretion needed investigation.

9.2. The diurnal rhythm of cortisol concentrations

As has been discussed in the introduction, early morning cortisol as often measured in clinical practice is not a particularly revealing investigation. Loss of the diurnal plasma cortisol rhythm has been described in some tuberculosis patients (Sarma, Chandra et al. 1990). Normal cortisol secretion observes a biorhythm with an early morning peak and an evening trough, it is this evening nadir which is lost in some studies. This has potentially important immunological consequences, outlined in the introduction, as T-cells are sensitive to local glucocorticoid milieu, even in the short term.

9.2.1. Methods

Patients with TB, cured patients and healthy volunteers recruited as described above were issued with saliva collection tubes (Salivette, Sarstedt, Leicester, UK). Specimens were collected at four-hourly intervals for 24 hours, once before and then again during treatment for tuberculosis. Specimens were briefly stored at 4° before being centrifuged at 4000 rpm for ten minutes and frozen at -20^{0}. These were subsequently analysed for salivary cortisol by radioimmunoassay (Harbuz, Rees et al. 1992) .

9.2.2. Results

Four hourly saliva collections for cortisol revealed an increased concentration of salivary cortisol at 2000h in samples from the patients with active tuberculosis (Table 7-1, Figure 9-1) This abnormality was found in the samples taken before initiation of antituberculous therapy, but was not significant after 3 days of treatment, or in the group with cured tuberculosis. Moreover, a single abnormal result must be interpreted with caution where multiple related data are collected in a time series. Such data are subject to the Bonferroni correction, where more rigorous critical significance is applied. The p value considered significant is $p<0.05/n$. By these criteria, the 8pm value among patients suffering from tuberculosis was not significant.

Both before and during treatment the patients with active TB had the lowest peaks and highest nadirs of cortisol, i.e. they have the smallest diurnal rhythms. However this was not statistically significant.

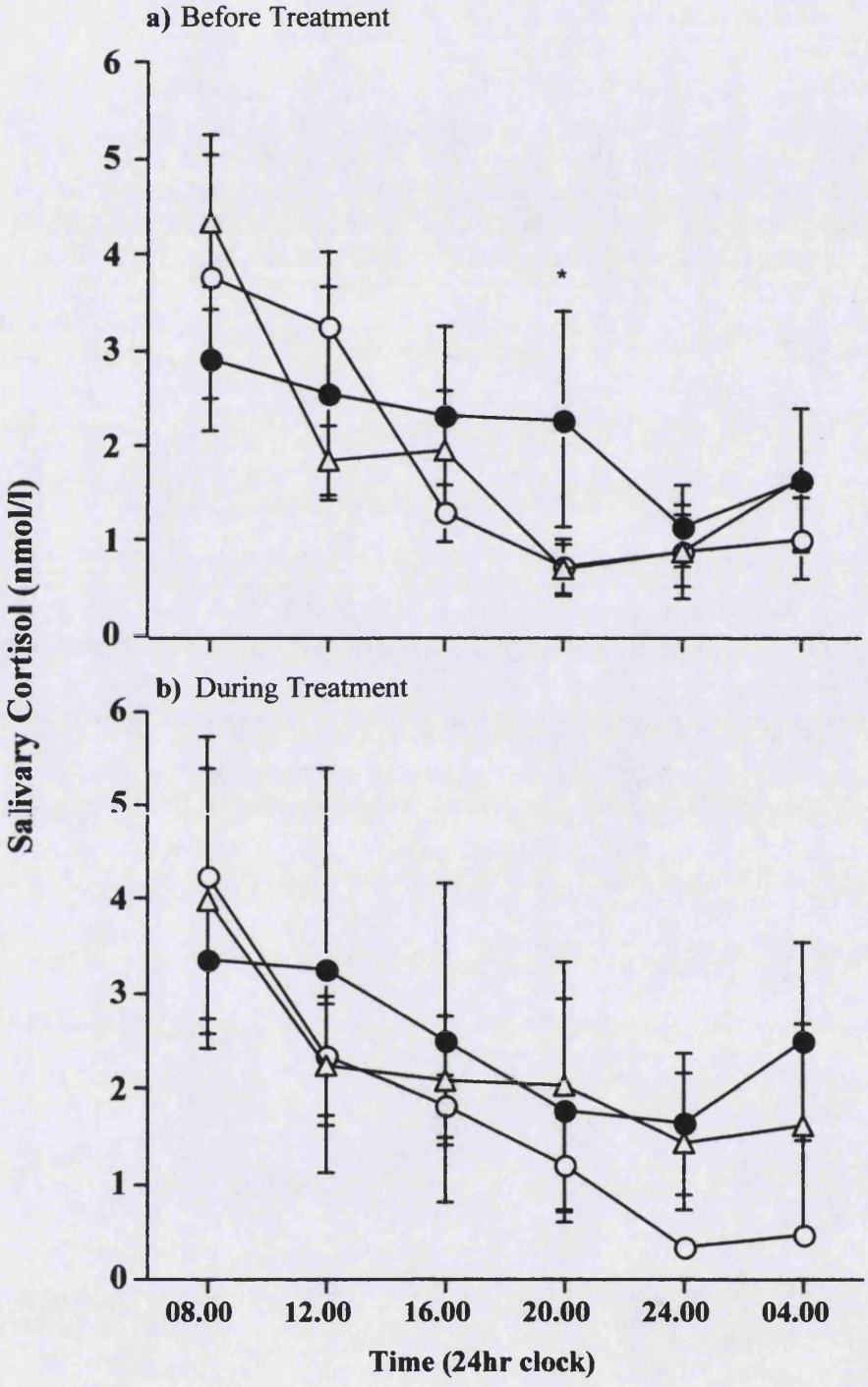


Figure 9-1 Diurnal variation of salivary cortisol, mean ± SEM. See over for key.

*Key to figure 9-1: (a) before antituberculous therapy. ●: Patients with tuberculosis, n=14; Δ: Patients cured of tuberculosis, n=13; O: Healthy volunteers, n=11; and (b) during antituberculous therapy. ●: Patients with tuberculosis, n=12; Δ: Patients cured of TB, n=11; O: Healthy volunteers, n=10. * indicates significantly elevated value in patients with tuberculosis. ANOVA before therapy between groups $p<0.05$, significant between acute tuberculosis and both other groups to $p<0.05$. ANOVA during therapy not significant.*

9.2.3. Discussion

These data suggested that there may have been some disturbance in diurnal rhythm of salivary cortisol secretion before treatment in patients with acute pulmonary TB. This corresponds with earlier reports suggesting disturbance of plasma cortisol diurnal rhythm (Sarma, Chandra et al. 1990). However, the disturbance disappeared after three days of treatment and hospitalisation. Only the patients with TB were hospitalised. The stress of hospitalisation is known to temporarily disturb this sensitive biorhythm. This was therefore felt to be the most likely cause of the temporary abnormality. A normal diurnal rhythm implies, but does not conclusively prove, an intact HPAA.

9.3. Responsiveness of the adrenal to ACTH

The response of the adrenal to exogenous synthetic ACTH is a standard endocrinological test of healthy adrenal output. This investigation has led to some confusion in the literature, in that some investigators have reported subnormal adrenal responses in patients with TB. The definition of abnormality has not been uniformly applied by all investigators; some considered a rise of less than 200nmol/l to be subnormal, where others used a rise of 300nmol/l (see

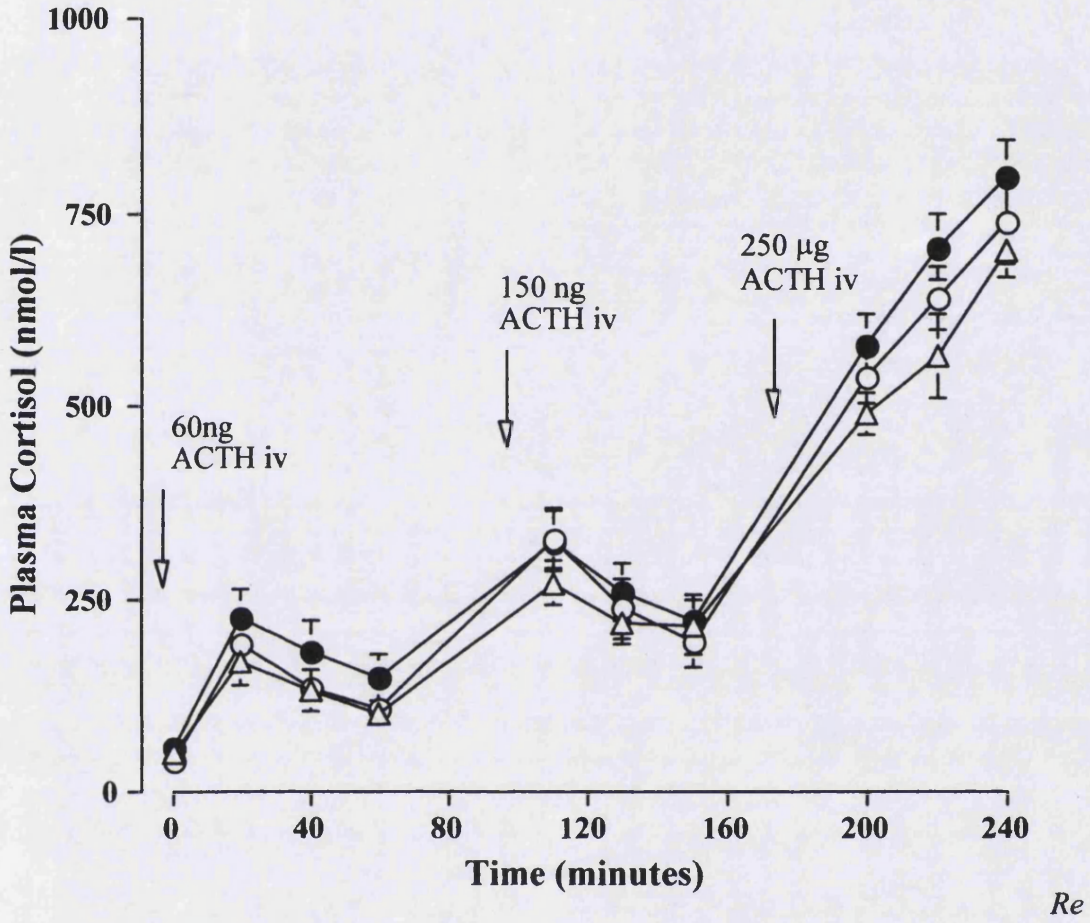
section 5.10). No previous investigator has published results of the investigation after adrenal suppression. The investigation may anyway be flawed, in that the standard dose of synacthen used (250μg) is supraphysiological by an order of magnitude.

9.3.1. Methods

Patients and controls were recruited as above. To test for the presence of even a subtle change in adrenal responsiveness to physiological levels of ACTH it was first necessary to suppress endogenous cortisol production by administering synthetic glucocorticoid. Having achieved this suppression of by administration of dexamethasone 2mg po 23.00 on the preceding evening, subjects received boluses of 60ng, 150ng and 250μg of ACTH at 90 min intervals, beginning at 09.00 with patients rested and supine. Plasma was collected at t=0, 20, 40 and 60, 110, 130, 150, 200, 220 and 240 minutes, i.e. before the first dose of synacthen and at 20, 40 and 60 minutes after each succeeding dose. Cortisol was then measured by radioimmunoassay (Harbuz, Rees et al. 1992).

9.3.2. Results

The adrenals of patients with acute tuberculosis responded normally to the physiological challenges (60ng and 150ng) and also to the supraphysiological challenge (250μg) (Figure 9-2).



Re

Figure 9-2 Serum cortisol responses, mean ± SEM, to sequential ACTH stimulation tests following dexamethasone 2 mg.

Key to figure 9-2: ●: Patients with tuberculosis, n=11; Δ: Patients cured of tuberculosis, n=11; O: Healthy volunteers, n=11. No significant differences between groups.

9.3.3. Discussion

The normal response to both physiological and standard doses of ACTH after dexamethasone suppression suggests that the adrenal is able to respond adequately in pulmonary TB. This is a very important negative finding, particularly taken in conjunction with the other normal HPAA investigations and the previously inconclusive and contradictory investigations performed by other groups. Tuberculosis is a disease which can infect any organ or tissue, and the adrenals are no exception. Tuberculous infection of the adrenals would lead to defective secretion of cortisol in response to ACTH, particularly at threshold doses after suppression with dexamethasone. The finding is also of interest given the abnormality of cortisol metabolism outlined above. Increased recruitment of cortisone, as demonstrated by increased cortisol:cortisone ratio, effectively means a longer half-life for cortisol. The predicted outcome of this would be adrenal suppression and disturbance of the sensitivity of the adrenal to threshold ACTH dosage. This does not appear, from these data, to be the case.

9.4. Response of the pituitary to CRH

Measurements of the response of the pituitary and adrenal to exogenous CRH have not previously been published. It is, however a crucial investigation in establishing the full function of the HPAA in chronic inflammatory states.

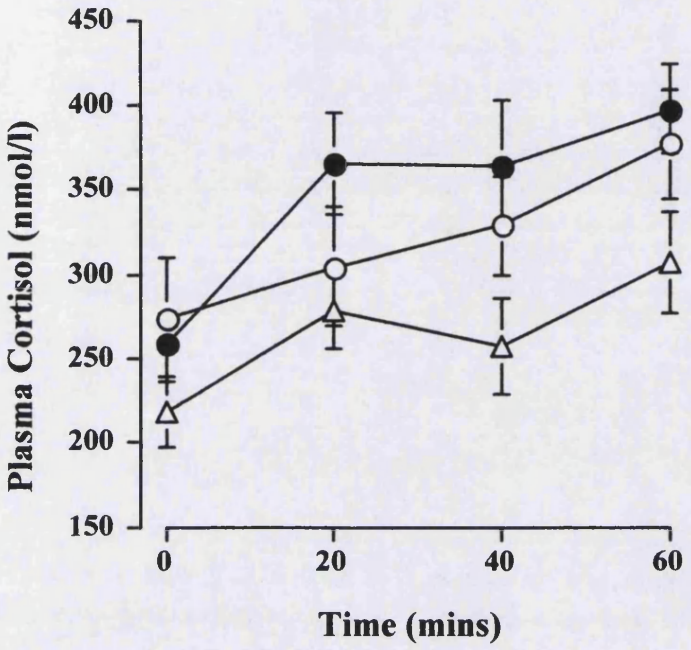
9.4.1. Methods

Patients and controls were recruited as above. Subjects were rested and supine during the investigation. 100 μg CRH (Ferring Pharmaceuticals Ltd. Feltham Middlesex UK) was injected at 0900 h and blood was withdrawn for 60 min from an intravenous cannula for

cortisol and ACTH. Plasma for ACTH assay was separated in a refrigerated centrifuge (4°) within ten minutes of being drawn. The subjects were not dexamethasone suppressed before this test was performed. Serum (Harbuz, Rees et al. 1992) and cortisol and serum ACTH (Cunnah, Jessop et al. 1987) were measured by radioimmunoassays.

9.4.2. Results

There were no significant differences between the groups in the increases in cortisol or ACTH which were observed following CRH (Figure 9-3 a and b).



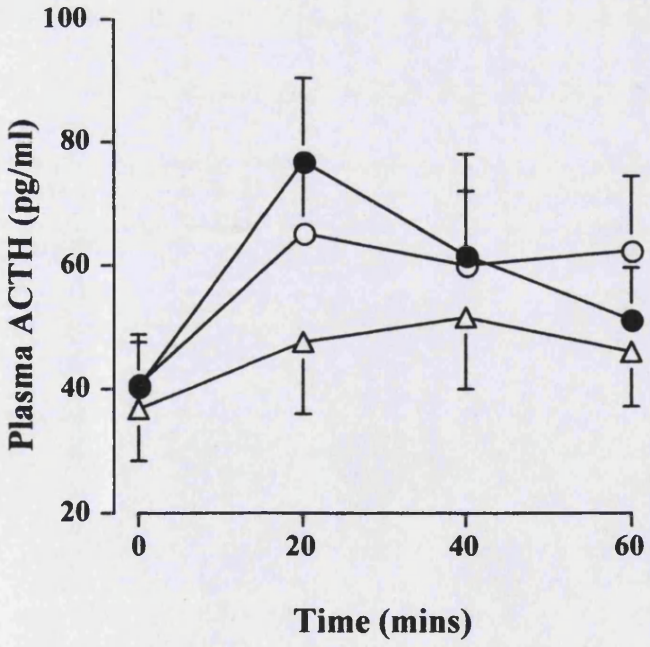


Figure 9-3 (a) Cortisol and (b) ACTH responses to CRH administered at time 0, mean ± SEM.

Key to figure 9-3: ●: Patients with tuberculosis, n=12; Δ: Patients cured of tuberculosis, n=11; O: Healthy volunteers, n=12. No significant differences between groups.

9.4.3. Discussion

Taken with the diurnal rhythm, normal 24-hour urinary cortisol output and response to ACTH the unremarkable CRH tests suggest an intact HPAA in patients with pulmonary tuberculosis. It should be noted that in rodents in some chronic inflammatory conditions the HPAA is disturbed in ways that would not be detected by this test (Chowdrey, Larsen et al. 1995), where the neurotransmitter secreted by the hypothalamus is arginine-vasopressin rather than corticotrophin. This hypothesis was not tested; however the normality of the diurnal rhythm of cortisol suggests that the CRH limb of the HPAA, which is under higher diurnal control, remains intact.

9.5. Suppression by dexamethasone

Suppression of the endogenous secretion of cortisol by dexamethasone was carried out for two reasons: to demonstrate that normal suppression of autonomous secretion is intact in these patients, and to suppress adrenal activity prior to stimulation with threshold doses of exogenous ACTH.

9.5.1. Methods

Patients and controls were recruited as above. Dexamethasone 250μg and then 2mg were administered at 23.00 on the days before the tests. Blood was drawn from intravenous cannula after 30 minutes supine rest. Serum cortisol was measured by radioimmunoassays (Harbuz, Rees et al. 1992). Serum dexamethasone was measured by gas chromatography and mass spectrometry (Best, Nelson et al. 1997).

9.5.2. Results

The degree of suppression of 0900h cortisol levels following consumption of 250μg dexamethasone at 2300h the previous night was similar in all groups (Table 7-1). Similarly suppression of 9.00 am cortisol levels to less than 50nmol/l was achieved in all groups following consumption of 2mg dexamethasone (Table 7-1). The concentrations of plasma dexamethasone achieved at each dose did not differ between groups (Table 7-1).

9.5.3. Discussion

Autonomous or adenomatous cortisol hypersecretion is a potential cause of cortisol excess. While this is an unlikely explanation of excess cortisol to cortisone ratio in these patients, given that diurnal rhythm of salivary secretion is effectively normal and that total urinary cortisol excretion was not elevated, it is clearly important to exclude the possibility. Normal suppression of cortisol secretion, particularly given the comparable plasma dexamethasone achieved, effectively excludes that explanation.

9.6. Renal 11-β HSD 2 function in TB

As discussed in the introduction, renal mineralocorticoid receptors are not specific for aldosterone or similar steroids. Cortisol itself binds to them with even greater affinity, and the role of renal 11-β HSD 2 is to protect these receptors from inappropriate activation. In the event that these receptors were to be activated by cortisol, the consequence would be functional hyperaldosteronism with a sodium retaining, potassium losing state (the opposite of that usually seen in pulmonary TB); as well as suppression of aldosterone and plasma renin activity by pressure and salt sensitive receptors in the juxta-glomerular apparatus of the kidney. Sensitivity of renal mineralocorticoid receptors to cortisol may therefore be inferred by these indices.

9.6.1. Methods

Subjects were recruited as above. Plasma was collected from rested, cannulated supine patients before any endocrine manipulations were performed and immediately separated in a refrigerated centrifuge (4°) and frozen (-20°) for subsequent analysis. Plasma renin activity and aldosterone were measured by radioimmunoassays (St. Mary's Hospital, Paddington, London, modifications of Menard and Catt 1972; Few, Chaudry et al. 1984).

9.6.2. Results

Sensitivity of renal mineralocorticoid receptors to endogenous cortisol, as judged by plasma renin activity, potassium, and aldosterone, was not different between groups (Table 7-1)

9.6.3. Discussion

These investigations suggest that the physiological activity of renal 11-β HSD 2 in protecting mineralocorticoid receptors from cortisol is intact. The inference is that the increase in cortisol: cortisone ratio described above is not due to downregulation of the enzyme at this site.

9.7. Summary of Activity of HPAA in TB

Intact function of the HPAA can effectively be established by three simple tests: normal 24-hour output of cortisol metabolites in the presence of normal cortisol binding globulin and normal diurnal rhythm of cortisol secretion. These tests were all intact in the patients with TB and cured patients when compared to healthy volunteers. The other tests of the HPAA – normal responses to CRH, ACTH, renal sensitivity to mineralocorticoids and dexamethasone suppression serve to confirm this fact. However, inflammatory conditions like TB tend to lead to release of pro-inflammatory cytokines which affect the HPAA. It is surprising therefore that there is no increase in overall cortisol output. Vagal afferents also signal directly to the brain in inflammatory situations, at least as far as the gut and peritoneal cavity are concerned (Laye, Bluthe et al. 1995; Bluthe, Michaud et al. 1996). This may also be true in the lung, and the expected outcome might be central stimulation of the HPAA with increased secretion of GC. However cortisol metabolism has clearly been demonstrated to be abnormal in patients with TB, in that total output is unchanged but a greater proportion of GC output is cortisol rather than cortisone. It was therefore necessary to look at peripheral cortisol handling to establish a site of a defect.

10.Cortisol/ cortisone ratios in human bronchoalveolar lavage.

10.1.1. Introduction

As has been stated, the interconversion of cortisol and cortisone is catalysed by 11β-hydroxysteroid dehydrogenases (11-β HSDs), which exist as at least two distinct isoenzymes. 11-β HSD type 2 is a high-affinity enzyme expressed in several sites, including the lung and blood vessels as well as the distal nephron where it acts as an exclusive 11-β dehydrogenase. Here it converts cortisol to cortisone and thereby protects renal mineralocorticoid receptors from inappropriate activation by cortisol (Edwards, Stewart et al. 1988; Funder, Pearce et al. 1988; Albiston, Obeyesekere et al. 1994; Kotelevtsev, Brown et al. 1998). 11-β HSD 2 is also present in human lung (Suzuki, Sasano et al. 1998) and in lymph nodes (Hennebold, Ryu et al. 1996). In contrast, 11-β HSD type 1 (Agarwal, Monder et al. 1989) is a low-affinity enzyme expressed in multiple tissues, including liver and lung (Berliner and Dougherty 1961; Schleimer 1991; Hubbard, Bickel et al. 1994; Rajan, Chapman et al. 1995). In most tissues, 11-β HSD 1 acts as a reductase, converting cortisone to cortisol and increasing local glucocorticoid receptor activation (Walker, Campbell et al. 1992; Jamieson, Chapman et al. 1995; Walker, Connacher et al. 1995; Bujalska, Kumar et al. 1997; Kotelevtsev, Holmes et al. 1997). The elevated ratio of cortisol to cortisone metabolites in tuberculosis could therefore reflect impaired 11-β dehydrogenase activity and/or enhanced 11-β reductase activity. Any change in cortisol metabolism could be important in two ways. Firstly, in tissues where 11-β HSDs are expressed there may be a net increase in local concentrations of cortisol. The

potential importance of this factor is illustrated by the observation that in mice infected with *Listeria monocytogenes*, the inhibition of 11-β HSDs with glycyrrhetinic acid increases their susceptibility to infection in a similar manner to the potent synthetic glucocorticoid dexamethasone (Hennebold, Mu et al. 1997). Secondly, decreased peripheral metabolism of cortisol could also explain the apparent paradox described above that circulating cortisol concentrations of cortisol are increased, while the adrenal gland is, according to some reports, smaller and less responsive to ACTH in tuberculosis. By prolonging the metabolic clearance rate of cortisol, negative feedback at the hypothalamus and pituitary may be enhanced and a compensatory reduction in cortisol secretion may occur with atrophy of the adrenal cortex.

An abnormality in the peripheral metabolism of cortisol in tuberculosis was therefore postulated, having established that the function of the hypothalamo-pituitary-adrenal axis is essentially normal. To test this hypothesis further specific metabolism of cortisol in the lung in patients with active tuberculosis and matched normal controls was assessed.

10.1.2. Methods: collection of BAL for steroid analysis

Two groups of 11 healthy volunteers and 13 patients with acute pulmonary tuberculosis participated in this study of cortisol metabolism in lung. Demographic details for all groups are recorded in Table 6-1. Diagnosis and exclusion criteria were as for all human studies as listed in section 6, as was the nature of consent of participating individuals. Patients with proven or suspected acute pulmonary TB or healthy volunteers attended for bronchoscopy at St Mary's Hospital, Paddington. Before the procedure blood was drawn for investigation of renal and hepatic function and flow cytometry as well as basal resting cortisol and cortisone. None of these individuals was being treated for pulmonary TB at the time of investigation.

Patients with tuberculosis providing specimens for bronchoalveolar lavage underwent the procedure before treatment was initiated.

10.1.3. Cortisol infusion

Where patients could be contacted in advance to obtain consent, an infusion of cortisol was commenced prior to the procedure. After an initial bolus of 3mg, an infusion of 4mcg/kg/min was infused for three hours. The infusion continued during the lavage. The purpose of the infusion was:

a) to establish a steady state of cortisol secretion and metabolism

b) to provide high concentrations of cortisol as enzyme substrate for 11βHSD

c) to obscure the effects of stress due to the procedure

Blood was drawn for plasma cortisol and cortisone post bronchoscopy and cortisol infusion.

Bronchoscopy was performed after sedation with intravenous midazolam 4-8mg and fentanyl 50-100mcg. Atropine 0.6mg was also infused as a bolus to prevent bradycardia, syncope and nausea during the procedure. Nasal passages, pharynx, vocal cords and bronchial tree were anaesthetised with topical lignocaine. Bronchoalveolar lavage was performed on diseased lung segments where so defined on chest X-ray, or right middle lobe where the chest X ray was normal. 250ml sterile normal saline was instilled with the end of the flexible bronchoscope wedged in a sub–segmental bronchus; fluid was then aspirated after minimal dwell time. Fluid was then immediately centrifuged at 4000 rpm for ten minutes to remove organic tissue that may have contained active glucocorticoid metabolising enzymes; this fluid supernatant was then frozen at -20^{o} for subsequent storage.

Before transportation for subsequent analysis, the supernatants were thawed and steroids were extracted on a C18 Sep-Pak (Waters Millipore, UK), and eluted with methanol. The eluted methanol was then dried under continuous nitrogen. Cortisol and cortisone were measured in bronchoalveolar lavage fluid by radioimmunoassay after separation by HPLC (Whitworth, Stewart et al. 1989).

10.1.4. Results

Plasma cortisol and cortisone concentrations were not different in patients with tuberculosis compared with healthy controls at baseline or during cortisol infusion. Cortisol/cortisone ratios in plasma were rather variable, but not different between groups. By contrast, cortisol/cortisone ratios in bronchoalveolar lavage fluid were higher in patients with tuberculosis (Figure 10-1). Cortisol/cortisone ratios in lavage fluid did not correlate with ratios or absolute levels of cortisol in plasma (BAL ratio versus plasma ratio $R= -0.01$ $p=0.95$; BAL ratio versus plasma cortisol concentration $R=-0.125$ $p=0.57$).

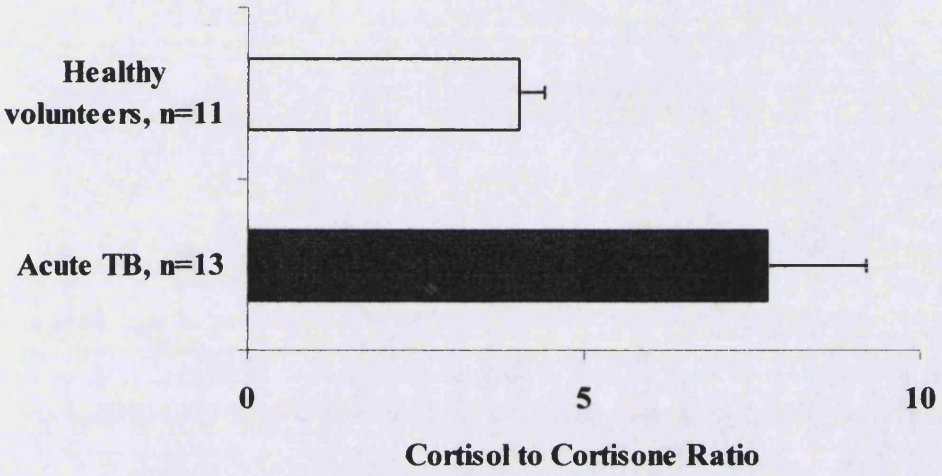


Figure 10-1Ratio of cortisol to cortisone in bronchoalveolar lavage in named groups, mean ± SEM.

Lavage was collected before treatment was commenced. Patients with acute tuberculosis (TB) had significantly elevated ratios compared to healthy volunteers (p=0.039, Mann Whitney U-test. See also Table 10-1).

	Acute tuberculosis	Healthy volunteers	p value Mann-Whitney U test
plasma cortisol before lavage (nmol/l)	542±66	618±117	0.64
plasma cortisone before lavage (nmol/l)	167±31	272±100	0.64
plasma cortisol during lavage (nmol/l)	1855±176	1814±138	0.21
plasma cortisone during lavage (nmol/l)	267±65	322±64	0.66
plasma cortisol/cortisone ratio during lavage	27.3±14.7	17.4±8.2	0.12
Lavage fluid cortisol (nmol/l)	20.8±4.5	14.43±2.6	0.34
Lavage fluid cortisone (nmol/l)	3.65±1.0	4.16±0.9	0.43
BAL cortisol: cortisone ratio	7.73±1.48	4.05±0.38	0.039

Table 10-1 Biochemical results from bronchoalveolar lavage study

10.1.5. Discussion

Against the background of normal functioning of the hypothalamo-pituitary-adrenal axis described in the chapters above, the changes observed in peripheral, pulmonary cortisol metabolism are striking. The previous finding that metabolism of cortisol is abnormal in patients with active tuberculosis is confirmed (Rook, Honour et al. 1996), and this abnormality appears, from these data, specific to tuberculous lung infection (in that patients with acute pneumonia were unaffected) and reversed by curing the tuberculous infection, as

reflected in urinary ratio (section 7.1.3). These data further suggest which isoenzyme of 11-β HSD is responsible for this change and where the abnormality arises.

Whether the increased ratio of cortisol/cortisone metabolites reflects impaired renal 11-dehydrogenase activity of 11-β HSD 2 or enhanced 11-β HSD 1 activity is unclear. In our experiments, the change was not associated with evidence of cortisol-dependent activation of mineralocorticoid receptors (plasma renin activity and aldosterone were not different in our patients with tuberculosis). This suggests that renal 11-β HSD 2 activity, at least, is unaffected. However, that might still be the case in a local disturbance of enzyme activity in the lung of the sort postulated here. It was associated with a relative increase in 5β-reduced metabolites (see Table 7-1) rather than the poorly understood decrease in 5β-reductase activity which accompanies congenital and acquired defects in 11-β HSD 2 (Walker and Edwards 1994). Moreover, the increased cortisol/cortisone metabolite ratio was associated with enhanced conversion of an oral dose of cortisone to cortisol in peripheral serum (see p 61). The latter test is normal in patients with congenital 11-β HSD 2 deficiency (Stewart, Corrie et al. 1988) and after 11-β HSD 2 inhibition by liquorice (Walker and Edwards 1994) but is impaired after administration of carbenoxolone (Stewart, Wallace et al. 1990; Walker and Edwards 1994), which inhibits both 11-β HSD 2 and 11-β HSD 1. The increased conversion of cortisone to cortisol therefore may be a reflection of enhanced 11β-reductase activity of 11-β HSD 1. However the relative impact of large organs such as lung, which receives the entire right sided cardiac output in each cardiac cycle, is likely to be relevant. Other large organs such as liver may also be extremely important.

There is substantial expression of both 11-β HSD 1 and 11-β HSD 2 in the lung (Escher, Frey et al. 1994; Hubbard, Bickel et al. 1994; Rajan, Chapman et al. 1995; Escher, Galli et al. 1997;

Suzuki, Sasano et al. 1998). Cortisol and cortisone have previously been quantified in BAL by Gas Chromatography Negative Ion Chemical Ionization Mass Spectrometry (Hubbard, Bickel et al. 1994). In this study BAL cortisone was measured as 1.05 nmol/l, cortisol as 0.42 nmol/l. Using HPLC and RIA cortisol and cortisone were measured in bronchoalveolar lavage fluid. The discrepancy in the absolute concentrations obtained (Table 10-1) may be attributed to the supraphysiological dosage of cortisol, which we infused for reasons outlined above. Crucially, despite there being no difference in circulating cortisol concentrations or cortisol/cortisone ratios between groups, the patients with active pulmonary tuberculosis had higher cortisol/cortisone ratios in bronchoalveolar lavage fluid. It is not possible to distinguish the influence of pulmonary 11-β HSD 2 and 11-β HSD 1 activities on the basis of these measurements alone, but for the reasons above, this is most likely to reflect local up-regulation of 11-β HSD 1 activity. The resultant enhanced conversion of inactive cortisone to active cortisol may promote local glucocorticoid receptor activation (Walker, Connacher et al. 1995; Kotelevtsev, Holmes et al. 1997) and thereby contribute to the inefficacy of Th1-mediated immunity which permits progressive tuberculosis. When T lymphocytes are recruited in the presence of raised concentrations of glucocorticoid, they are biased towards a Th2 cytokine profile (Brinkman and Kristofic 1995; Ramirez, Fowell et al. 1996). This is important because, although the T cell response of tuberculosis patients is dominated by Th1 cytokine production (Barnes, Lu et al. 1993), there is abundant evidence for the simultaneous presence of an inappropriate Th2 component, manifested as specific IgE antibody (Yong, Grange et al. 1989), expression of mRNA for IL-4 in peripheral blood lymphocytes (Schauf, Rom et al. 1993) and release of IL-4 *in vitro* (Sanchez, Rodriguez et al. 1994). Experimentally, even a minimal Th2 component renders the Th1-mediated immunity ineffective in this disease, and increases cytokine-mediated immunopathology (Hernandez-Pando, Pavon et al. 1997).

The influence of altered cortisol metabolism on local glucocorticoid concentrations in the lung and elsewhere is not the only important inference from this study. Enhanced 11β-reductase activity will decrease the metabolic clearance rate of cortisol which, in the presence of a normal hypothalamo-pituitary adrenal axis, could increase negative feedback and suppress ACTH secretion. This mechanism could contribute to the adrenocortical atrophy (Hernandez Pando, Orozco et al. 1995) and reduced excretion of cortisol and adrenal androgen metabolites which has been observed previously in more advanced cases of tuberculosis (Rook, Honour et al. 1996). However, in the current study, cortisol and androgen secretion were not suppressed in patients with tuberculosis. It may be that an equilibrium is reached between a tendency for reduced cortisol secretion as a result of impaired metabolic clearance and a tendency for increased drive to CRH, ACTH, and cortisol secretion from increased production of cytokines such as TNF-α, IL-1, and IL-6 which stimulate the hypothalamo-pituitary-adrenal axis. Indeed it is a paradox that there is not a strikingly increased output of cortisol in an inflammatory disorder such as tuberculosis which is accompanied by massive release of these cytokines (Besedovsky, del-Rey et al. 1991). TNF-α and IL-1 activity could be modulated by the relative concentrations of their antagonists, sTNFr and IL-1Ra respectively. However no specific antagonist of IL-6 has yet been described. Altered metabolic clearance of cortisol may also explain previous observations of elevated circulating 0900h cortisol concentrations in tuberculosis (Sarma, Chandra et al. 1990; Post, Soule et al. 1994), since the duration of the decay of the normal morning peak of plasma cortisol will be prolonged, and 0900h plasma cortisol may be elevated as a result.

11. Involvement of Interleukin-6 in control of the Hypothalamo-Pituitary-Adrenal Axis in health and disease. Measurement of Interleukin-6 and soluble IL-6 receptor in urine.

11.1.1. Introduction

The abnormality of peripheral cortisol metabolism described so far could be mediated by a number of factors. Chief among these would be local cytokines, such as Interleukin-6 (IL-6). This is a likely candidate cytokine for a number of reasons.

a) Raised concentrations of IL-6 are expressed in alveolar macrophages in broncho-alveolar lavage in patients with pulmonary TB (Law, Weiden et al. 1996)

b) IL-6 is produced by peripheral blood mononuclear cells in TB (Saunders, Robson et al. 1993).

c) Pleural IL-6 levels correlate with (but are higher than) serum Il-6 levels. Both are higher in patients with TB than malignancy (Yokoyama, Maruyama et al. 1992).

d) Ingestion of MTB by human monocytes results in increased IL-6 secretion by those cells (Friedland, Shattock et al. 1993).

e) *M. tuberculosis* activates the interleukin 6 gene (Zhang, Lin et al. 1995).

f) Patients with TB with low IL-6 tend to die (Friedland, Hartley et al. 1995).

g) IL-6 is necessary to maintain sterilising immunity in mice infected with *M. tuberculosis* (Appelberg 1994).

h) In contrast to other cytokines such as TNFα which are active on the HPAA, there is no known IL-6 inhibitor.

Several cytokines are known to influence the regulation of release of glucocorticoids by action on the hypothalamus and/or the pituitary gland. The major cytokines involved are IL-1, IL-2, IL-6, TNF-alpha and interferon-tau. (Jones and Kennedy 1993). Of these, IL-1 is the most potent. (Besedovsky, del-Rey et al. 1991) However, lower doses of rhIL-1 alpha and rhIL-6 synergize to induce a response similar to IL-1 alone (Perlstein, Mougey et al. 1991). Elevated human plasma ACTH and cortisol levels promptly follow infusion of IL-6, peaking 4 h after administration (Spath Schwalbe, Born et al. 1994). IL-6 elevates ACTH secretion at about 1 h in rats. The effects of IL-6 on the hypothalamo-pituitary-adrenal axis are therefore mediated through the hypothalamic paraventricular nucleus (Kovacs and Elenkov 1995), as destruction of paraventricular nuclei completely prevents this increase of ACTH plasma levels after IL-6 injection. This may, as in IL-1, be signalled via vagal afferents rather than by crossing the blood-brain barrier.

Il-6 is biologically inactive. Two IL-6 molecules conjugate into a hexameric subunit with two molecules of its soluble receptor, sIL-6R and a further protein, gp130, before there is any activity (Ward, Howlett et al. 1994). Therefore urinary production of the soluble receptor sIL-6R was measured as well as IL-6.

11.1.2. Methods

In order to find out whether IL-6 and S-IL-6R concentrations were elevated in the urine of patients with TB, and whether any such effect was disease specific or constitutive, urinary estimation of this cytokine was performed by ELISA on 35 individuals; 10 healthy volunteers, 11 with pulmonary TB, 8 with cured pulmonary TB and five with acute non-tuberculous pneumonia. Assays were performed in duplicate and a mean reading was obtained. 24 h urine

volumes had been documented at time of collection, which enabled estimation of complete daily output of IL-6 and S-IL-6-R

IL-6

A commercial kit manufactured by Milenia™ (MKL61) was used for human IL-6 assay by sandwich ELISA. The microplate contained wells coated with IL-6 monoclonal antibodies to which samples and standards were added. After a washing step, a horseradish peroxidase-labelled monoclonal antibody, directed against another epitope of the IL-6 molecule, was added. During a 2-hour incubation, a sandwich complex consisting of the two antibodies and the IL-6 is formed. Unreacted material is then removed by a washing step. A chromogenic substrate (3,3'. 5,5'-tetramethylbenzidine, TMB) reactive with the enzyme level was added. Colour development was terminated with acidic stop solution after 30 minutes. The resulting colour, read at 450nm, was measured relative to an extraction curve derived from serial dilutions of the calibrator supplied with the kit.

SIL-6-R

Human S-IL-6 R was estimated using a Quantikine™ (catalogue number DR600) sandwich immunoassay. This assay uses a similar technique to the IL-6 assay; samples are incubated on plates in wells coated with immobilized antibody. After a washing step, an enzyme -linked polyclonal antibody specific for s-IL-6R is added to the wells, washed and a substrate solution added. The colour develops in proportion to the amount of sIL-6 R bound in the initial step.

11.1.3. Results – IL-6

A number of individuals in each group, apart from the cured TB group, had urinary IL-6 concentrations below the detection range of the standard curve (15.6 - 500 pg/ml). The detected range was 0-143.88 pg/ml. There was no significant different between groups (ANOVA).

	24hr IL-6 (ng/24 hrs)	SE
HV n=11	21.78	7.93
TB n=12	53.65	21.39
CTB n=8	88.75	16.69
DC n=5	31.14	19.18

Table 11-1 24 hour IL-6 output in TB patients and controls. DC = Disease Controls; patients with acute non-tuberculous pneumonia.

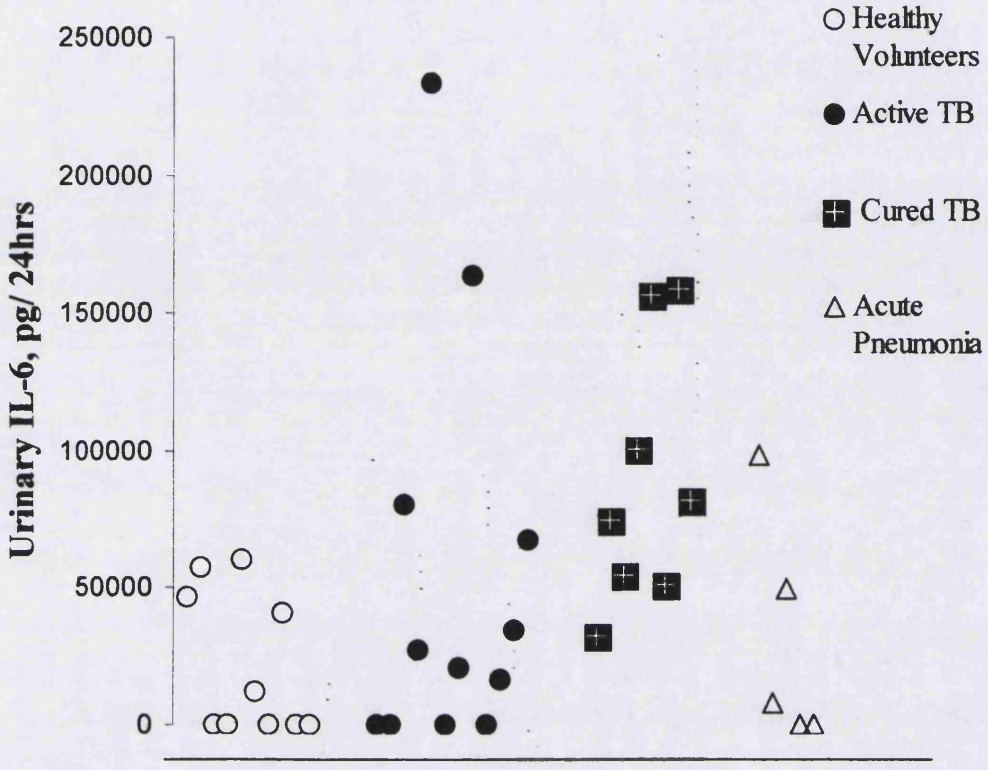


Figure 11-1. 24 hour output of IL-6 in individuals with TB and controls

11.1.4. Results – SIL-6-R

Two individuals had SIL-6R concentrations above the assay detection range (31.2-1000 pg/ml); none had levels below the threshold range. There was no significant difference between groups (ANOVA).

	24hr sIL-6R (ng/24 hr)	SE
HV n=11	542.71	7.93
TB n=12	376.02	21.39
CTB n=8	471.36	16.69
DC n=5	300.42	130.73

Table 11-2 24 hour S-IL-6R output in TB patients and controls.

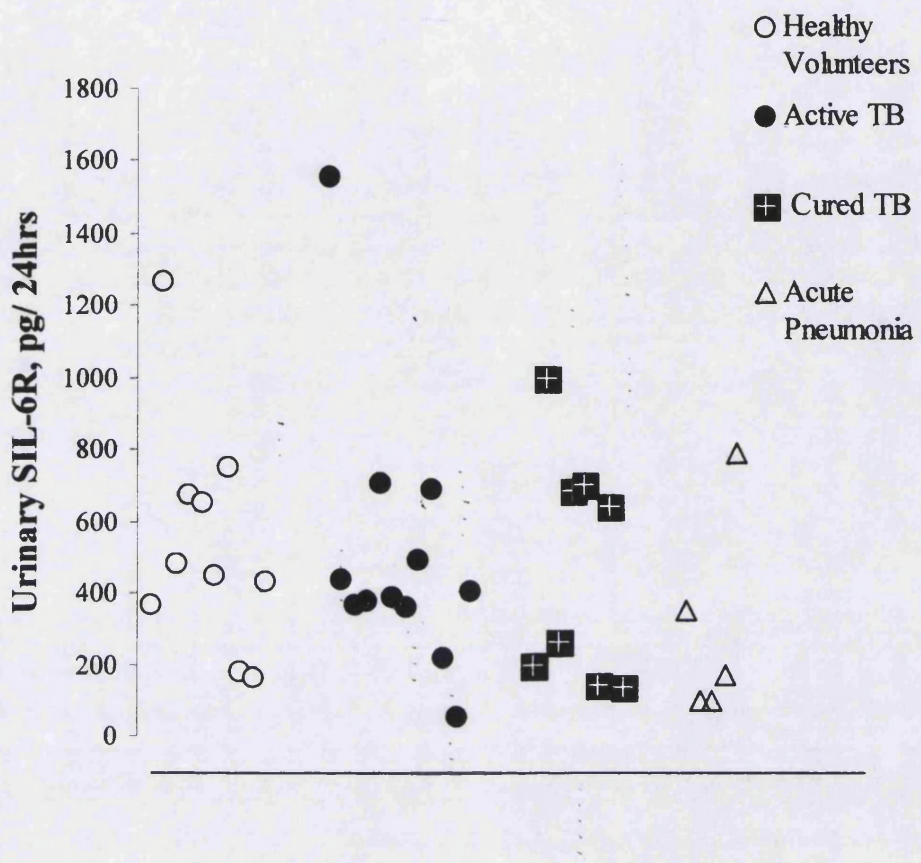


Figure 11-2 24 hour output of S-IL-6R in individuals with TB and controls.

There was no correlation between IL-6 and SIL-6R in any of the groups.

11.1.5. Conclusions

In this study, there was no difference between patients with pulmonary TB, healthy volunteers, patients cured of TB and patients with acute non-tuberculous pneumonia in urinary IL-6 or S-IL-6 R production in terms of urinary concentration or 24 hour output. However, IL-6 assay may not have been optimal in that a number of individuals had IL-6 concentrations below the range of the assay; this may indeed represent the physiological state. While IL-6 and SIL-6R are undoubtedly involved in inflammation and the pathological response to

tuberculous infection and stimulation of the HPAA, and IL-6 is the only pro-inflammatory cytokine with no known inhibitor in TB, the relationship would seem to be more complex than might be simply detected by estimation of 24-hour urinary output. The absence of clear evidence of elevated 24-hour output of IL-6 and s-IL-6R is therefore interesting given that there was no apparent increase in 24-hour cortisol output in the patients with TB. Repeat of the experiment with an assay with a wider detection range might clarify the issue further.

12.Intracellular cytokine production in human blood and lungs in health and disease

12.1. Introduction

The classical model of the immune response to tuberculosis involves a proliferation of Th1 cells which produce IFN with subsequent activation of macrophages. Th1 cells are present in the bronchoalveolar lavage of patients with tuberculosis (Robinson, Ying et al. 1994) and others have found Th1 cells in pleural fluid from tuberculous pleuritis (Barnes, Lu et al. 1993). Abnormalities in IFN receptors predispose to persistence of mycobacterial disease (Newport, Huxley et al. 1996). T cells are required for efficient activation of peripheral blood gamma delta T cells by *M. tuberculosis* (Pechhold, Wesch et al. 1994). Increased production of IL-12 by peripheral blood monocytes, a stimulus to Th1 cell differentiation, has been shown in tuberculosis (Cooper, Roberts et al. 1995). There is also limited data to show that patients who mount a poor response to tuberculosis as manifested by severe disease have a greater Th2 response than tuberculin positive healthy controls (Sanchez, Rodriguez et al. 1994).

Corticosteroids are well recognised to suppress immune function by downregulating macrophages and influencing T-cell maturation (Rook, Steele et al. 1987). Although both mature Th1 and Th2 cells are affected by corticosteroids, there is increasing data to suggest that the hormone cortisol differentially affects T-cell differentiation in favour of Th2 cells as opposed to Th1 cells. In other words glucocorticoids drive a Th2 response, although if present

in high enough concentration, they can suppress Th2 effector function. (Brinkman and Kristofic 1995; Ramirez, Fowell et al. 1996). In the context of tuberculosis these cells are tissue damaging (Yong, Grange et al. 1989; Schauf, Rom et al. 1993; Cooper, Bouic et al. 1995). The abnormality of pulmonary cortisol metabolism described in chapter 7 would lead to locally increased concentrations of active cortisol at the expense of inactive cortisone. It was therefore important to establish whether this relative excess of cortisol leads to alteration in relative numbers of cells secreting Th1 or Th2 cytokines.

The cytokines which have traditionally been considered to represent polarisation of Th1 and Th2 responses are IL-2 and IFNγ (both Th1) and IL-4 (Th2) (Mosmann, Cherwinski et al. 1986; Modlin 1994). These cytokines were therefore selected for analysis, as well as IL-5 as a further Th2 cytokine (Cherwinski, Schumacher et al. 1987). As has been explained in the introduction, the situation is complicated by the involvement of other glucocorticoid sensitive cytokines associated with Th2 such as IL-10 and TGFβ.

12.1.1. Why flow cytometry?

Flow cytometry is the measurement of cells in a system which has been designed to deliver particles in single file past a point of measurement. Although, in theory, many types of measurement could be made, in practice the term is applied to instruments which focus light on to cells and record their fluorescence and the light scattered by them. Electronic cell volume and absorbed light (and therefore the granularity of the cells) may also be measured. The power of flow cytometry lies in the ability to measure several parameters on tens of thousands of individual cells within a few minutes. The method can therefore be used to

define and to enumerate accurately sub–populations, such as subsets of lymphocytes secreting specific cytokines. Lymphocytes can be identified from specimens of whole blood and BAL by their size and granularity. In these experiments, five parameters were measured on up to 10 000 cells gated for size and granularity to identify them as lymphocytes per specimen, in human bronchoalveolar lavage and blood.

Flow cytometry makes a measurement on each cell individually so that, if a small subset of cells has a high value (e.g. CD4 cells secreting IL-2), this feature will be recorded. Such a subset would not be detected biochemically. However, if a small subset has a low value this would not be well recorded, and therefore this detection method is not suited to detection of secretion of molecules of high biological activity in small quantity by a small number of cells.

12.1.2. Labelling of proteins for Flow cytometry

There are two classes of fluorophore used in flow cytometry - those which are covalently bound to other probes (almost invariably a protein) and those which bind non-covalently to structures within the cell. Labelled proteins may be used for immunofluorescence. Monoclonal antibodies may be labelled directly or indirectly. The latter is achieved either with a labelled antibody to immunoglobulin or by labelling the primary antibody with biotin and using fluorescently labelled streptavidin which has four binding sites for biotin (Polak and Van Noorden 1984; Ploem and Tanke 1987) . The most common fluorophore used to label proteins is fluorescein iso-thiocyanate (FITC). Labelling is achieved by reaction of the isothiocyanate with lysine residues on proteins. PE is a large protein and a different method has to be used. It can be joined directly to immunoglobulins with a linker molecule such as N-succinimidyl 3-(2-pyridyldithio) propionate. Alternatively it can be labelled with biotin and combined with a biotin- labelled antibody with either avidin or streptavidin as a bridge.

Directly labelled monoclonal antibodies and labelled anti-immunoglobulins were purchased directly from manufacturers (see Table 12-5).

Stimulation of committed T-cells to produce specific cytokines may be achieved by exposure to a combination of phorbol 12-myristate 13-acetate (PMA) and calcium ionophores such as ionomycin (Erard, Nabholz et al. 1985). T cells may also be stimulated by exposure to mycobacterial cellular products such as purified protein derivative (PPD). However exposure to such antigens would result in differentiation of T-cells along previously uncommitted paths. This set of experiments sought to establish which cytokines the cells were already committed to secreting.

Labelling of intracellular cytokines requires that they remain within the cell during stimulation and permeabilisation. Under normal circumstances cells stimulated to secrete any molecule would release them into the local environment. For the purposes of flow cytometry, the release of these cytokines may be prevented by the addition of the carboxylic ionophore monensin which is used to interrupt intracellular transport processes leading to an accumulation of the cytokine in the Golgi complex (Jung, Schauer et al. 1993).

12.1.3. Collection of Lavage and Blood Specimens for Flow Cytometry

Whole blood was collected immediately after bronchoscopy in bottles containing 1000 units mucous heparin and stored at 4° prior to stimulation (see below for data relating to delay in stimulation of T-cells). Lavage was collected as described above (Chapter 8.1.2). The fluid from lavage specimens was required for analysis of cortisol and cortisone. The specimens were therefore immediately centrifuged after collection and supernatants stored. The cellular residue was immediately washed and then resuspended in RPMI and 5% Foetal Calf Serum to ensure cell viability. These cells were stored in an incubator at 37° in 5% CO_2 overnight

before addition of stimulants (see optimisation step below, Table 12-4, Figure 12-7). Cells from patients with open pulmonary TB were handled in Category III isolation.

12.1.4. Effect of cortisol infusion on intracellular cytokine secretion

The essence of this thesis is the interaction between cortisol and immunological factors such as cytokine secretion by T-cells. As has already been described, cortisol can directly affect cytokine production by T-cells. For reasons outlined on p. 82, lavage specimens were collected after a bolus and then an infusion of cortisol. This might be expected to confound the interpretation of T-cell responses. This was not felt to be a problem for two reasons:

a) cortisol acts on intracellular cytokine secretion via an intracellular receptor which affects mRNA and protein synthesis. The duration of the cortisol infusion was a maximum of four hours; while this may or may not be long enough to down-regulate or up-regulate cytokine synthesis, more important is the fact that all individuals, including controls, were subjected to this intervention.

b) At least in the case of IL-4, stimulation by PMA and ionomycin activates cells that are *already committed* to a particular pattern of cytokine secretion.

12.2. Optimization of detection of intracellular cytokines:

12.2.1. PMA/ Ionomycin/ Monensin concentrations

Concentrations of phorbol 12-myristate 13-acetate (PMA) were optimised using whole blood and BAL T cells of healthy individuals, collected as described above. IFNγ was selected as the cytokine most readily expressed in healthy individuals in CD4 and CD8 cells. Stimulants

were prepared in doubling concentrations. Concentrations of ionomycin (Sigma I-0634) (2.0μMol) and monensin (Sigma M-5273) (3.0μMol) were as per manufacturers' instructions, and personal discussions with laboratory colleagues.

12.2.2. Results:

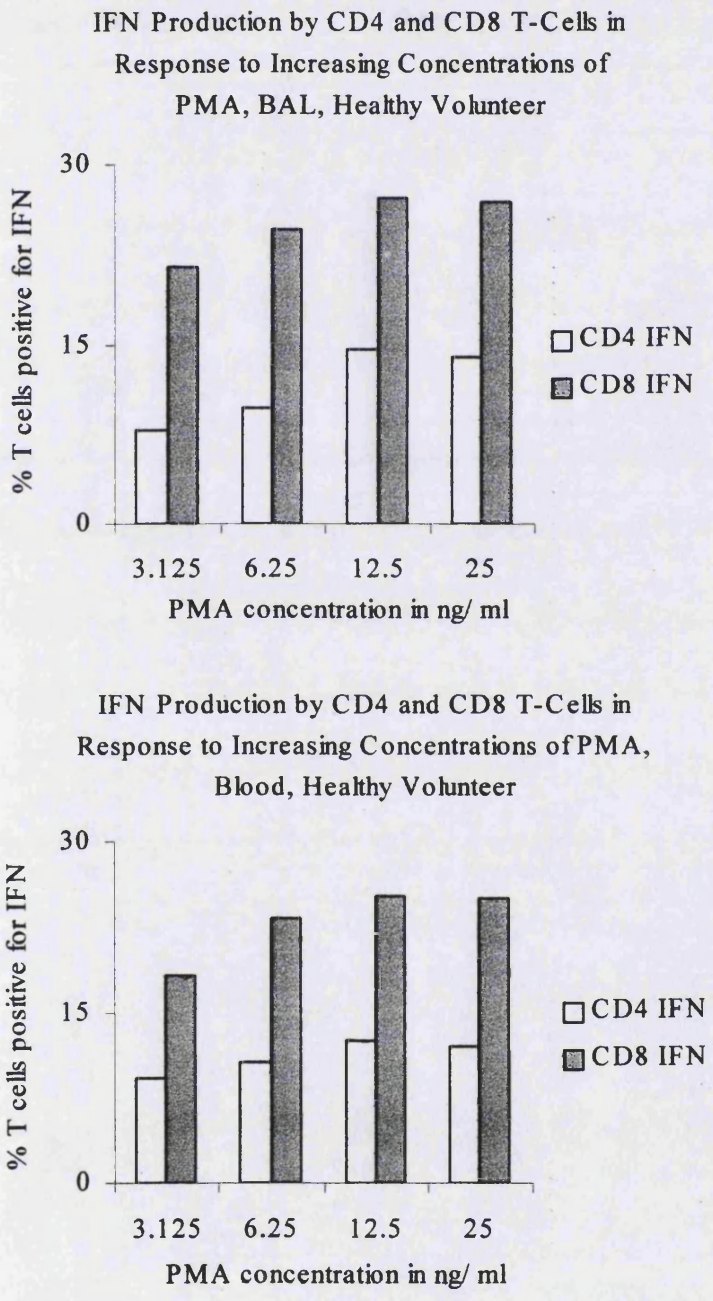


Figure 12-1. Optimisation of PMA concentrations.

IFN production peaked at a PMA concentration of 25ng/ml (12.5ng/ml when mixed with equal volume of blood/ BAL); this was therefore selected as the optimum concentration.

12.2.3. Optimisation of duration of stimulation

BAL and Peripheral blood cells were collected from a healthy volunteer as above. Cells were stimulated in RPMI the presence of previously optimised concentrations of PMA, ionomycin and monensin for 2, 6, 12 and 24 and 48 hours. Cells were then surface stained, lysed, permeabilised, exposed to intracellular cytokine and fixed; they were then run on a flow cytometer as described above.

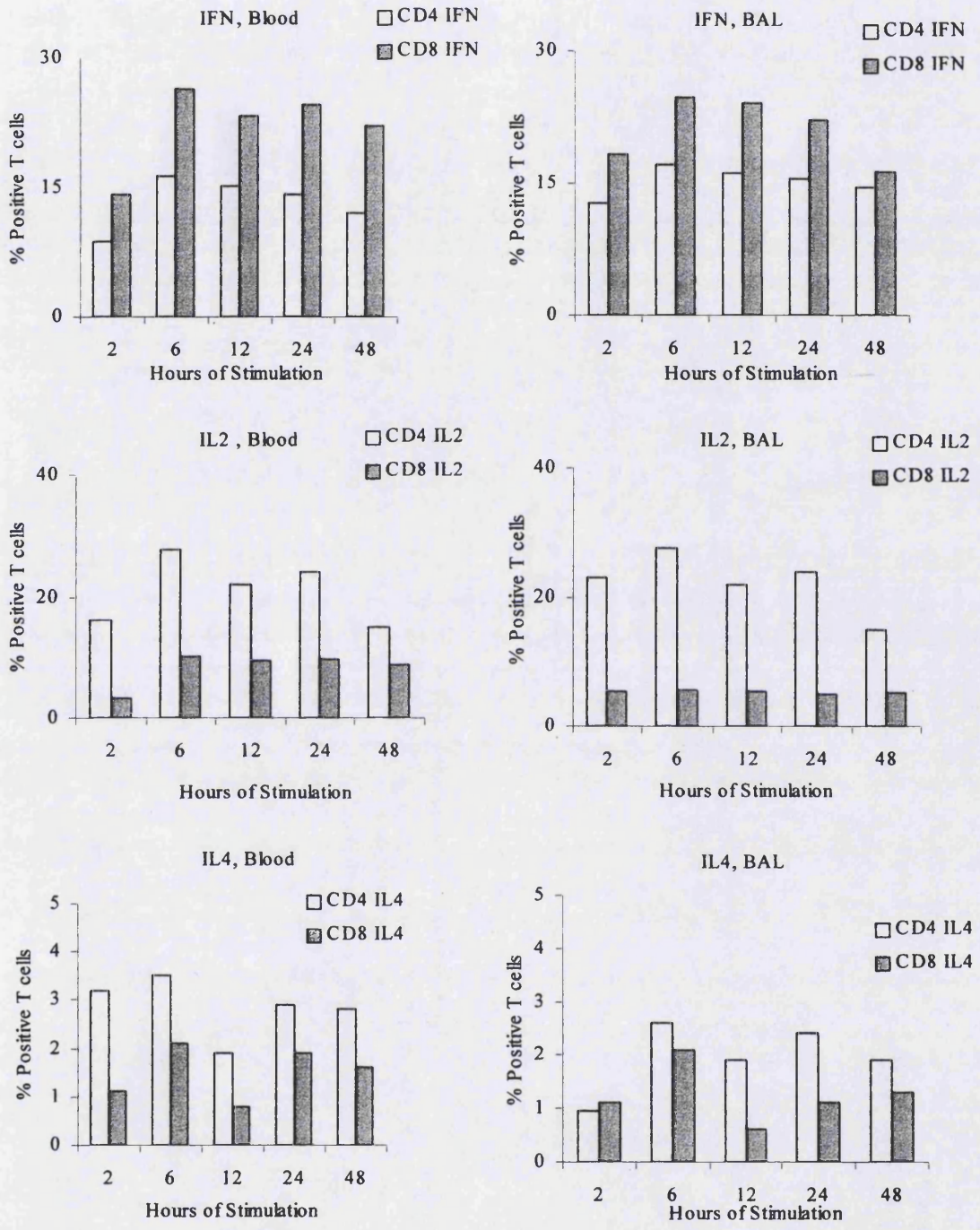


Figure 12-2 Optimisation of duration of stimulation of T cells with PMA and Ionomycin.

12.2.4. Discussion

The optimal duration of stimulation of T-cells was established to be 6 hours. Cells exposed to PMA for longer periods appeared to decrease the number of active T-cells, possibly through cell death.

12.2.5. Optimisation of isotype control concentrations

Convincing evidence of intracellular production of cytokines by lymphocytes requires the demonstration of negative controls. Two kinds of negative controls were used in these experiments; stimulated cells without any stain and stimulated cells stained with so-called isotype controls. These are the same colour stains (FitC, Cy5 and PE) as are conjugated to the monoclonal antibodies themselves. However, instead of being conjugated to monoclonal antibodies, they are conjugated to non-specific immunoglobulins of the same class. Binding of cells to these stains therefore corrects for the possibility of non-specific antibody binding inherent in any antibody: antigen binding system.

The concentrations of isotype stains used was determined by exposing blood and lavage cells stimulated in the presence of ionomycin and monensin to increasing concentrations of isotype stain. In higher concentrations non-specific binding also begins to occur; this is demonstrated by shift of the geometric mean of the bulk of cells when compared with unstained controls. The stain concentrations used were therefore determined to be the highest concentration that would not cause shift in the geometric mean. The volumes used are listed in Table 12-1.

12.2.6. Optimisation of intracellular cytokine stain concentrations.

In order to determine the optimal concentration of conjugated intracellular monoclonal antibody stain for each cytokine, both blood and bronchoalveolar lavage were stimulated in

the presence of ionomycin and monensin as described below, surface stained, lysed, permeabilised and exposed to increasing concentrations of individual stains. The concentrations were derived from manufacturer's instructions and were serially diluted and concentrated from this starting point. Monoclonal stains varied in initial concentration by weight according to supplier (see Table 12-1). The optimal concentration was considered to be that which gave highest percentage of stained cells (having subtracted isotype controls) without significant shift of the geometric mean of the unstained cells. Higher concentrations of monoclonal stains tend to bind non-specifically to cell surface receptors giving misleadingly false-positive results; this is indicated by shift in the geometric mean of fluorescence intensity.

Stain/ manufacturer	Catalogue number	Concentration	Vol added in µl / 100 µl cells
IL-2 FitC Becton Dickinson	340448	12.5µg/ml	10
FitC Isotype IgG1 BD	349041	50µg/ml	2.5
IFN FitC Pharmingen	18904A	0.5mg/ml	2.5
FitC isotype IgG2α Pharmingen	20604A	0.5mg/ml	2.5
IL 4 PE BD	340451	0.5mg/ml	1.25
IL 5 PE BD	18515A	0.5mg/ml	1.25
PE Isotype IgG1 BD	349043	0.5mg/ml	1.25

Table 12-1 Volume of intracellular cytokine added, catalogue numbers and concentrations

12.2.7. Results:

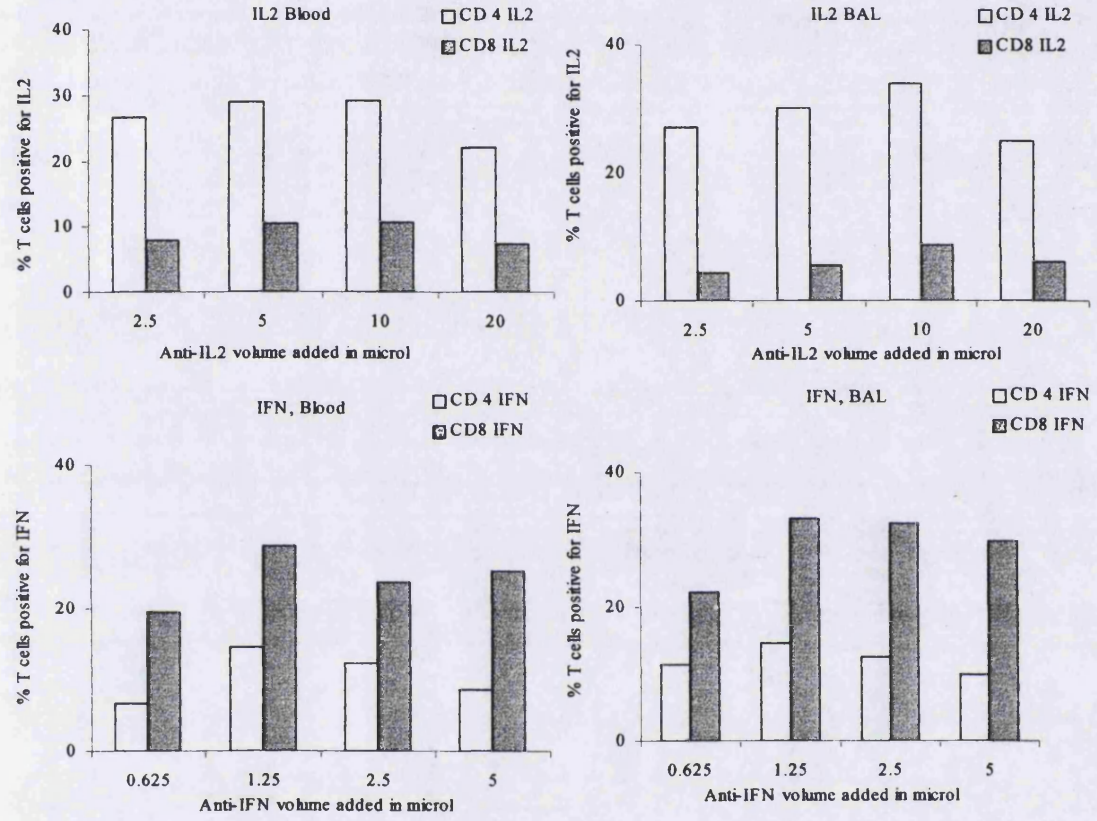


Figure 12-3 Optimisation of volume of Th1 intracellular cytokine antibody.

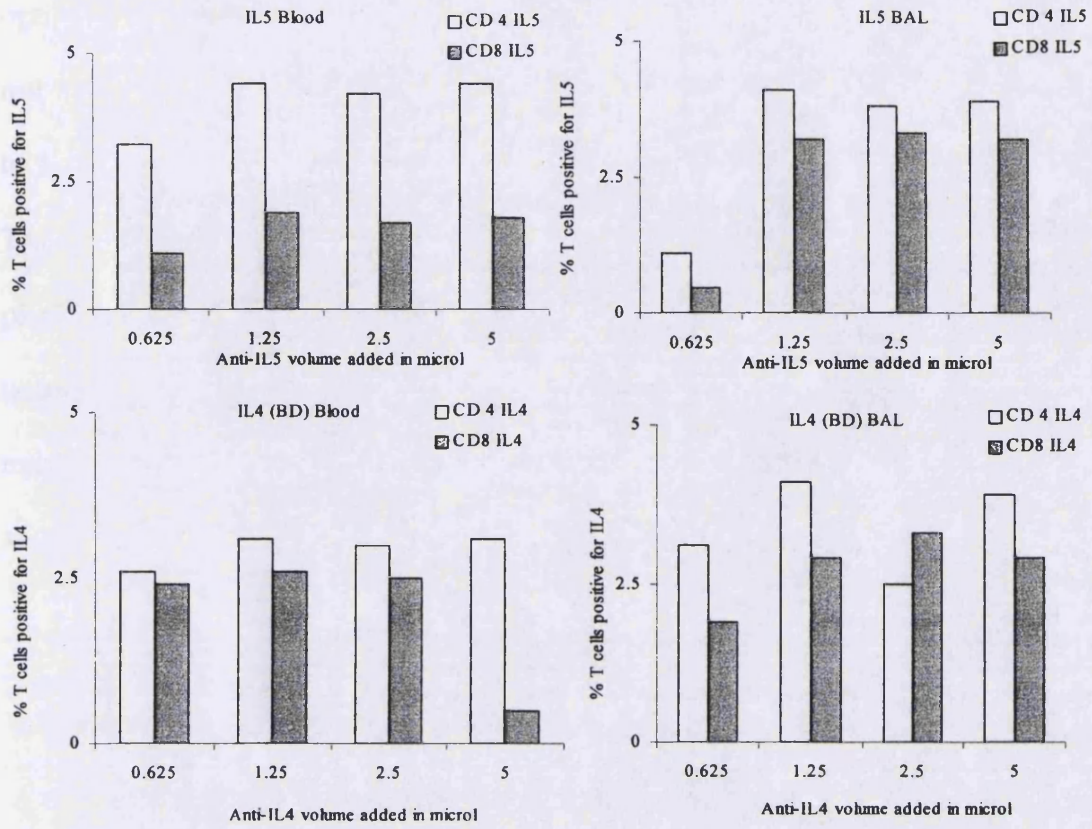


Figure 12-4 Optimisation of volume of Th2 intracellular cytokines

Serial numbers and manufacturers of individual stains are listed in Table 12-5, Table 12-6 and Table 12-7. Optimal concentrations of monoclonal stain for IFNγ, IL-2 and IL-5 were readily derived. Disparities between intracellular monoclonal stain volumes of anti-IL-2 FitC and volumes of FitC isotype are explained by the relative concentrations of stains as supplied by manufacturers; the eventual concentrations in mg/ml matched. However there were specific problems with IL-4 (Pharmingen), discussed below

12.2.8. IL-4 stain problems.

The monoclonal stain initially selected for demonstration of intracellular production was Pharmingen Anti-IL-4 fluorochrome PE conjugated (serial number 18505A). Attempts to

optimise this stain met with problems. Increasing concentrations of PE conjugated stain led not to the isolation by flow cytometry of a small distinct population of IL 4 positive cells, but to a shift in the geometric mean of the bulk of gated lymphocytes along the axis of detection. This made the analysis of the number of T-cells positive for IL 4 impossible. It is a phenomenon suggestive of non-specific binding to a cellular receptor. The phenomenon was noted by other investigators within the institution and was therefore presumed to be a fault in manufacture; this was subsequently confirmed by the supplier. The problem was resolved by switching to an alternative supplier (Becton Dickinson) and stain (Table 12-1).

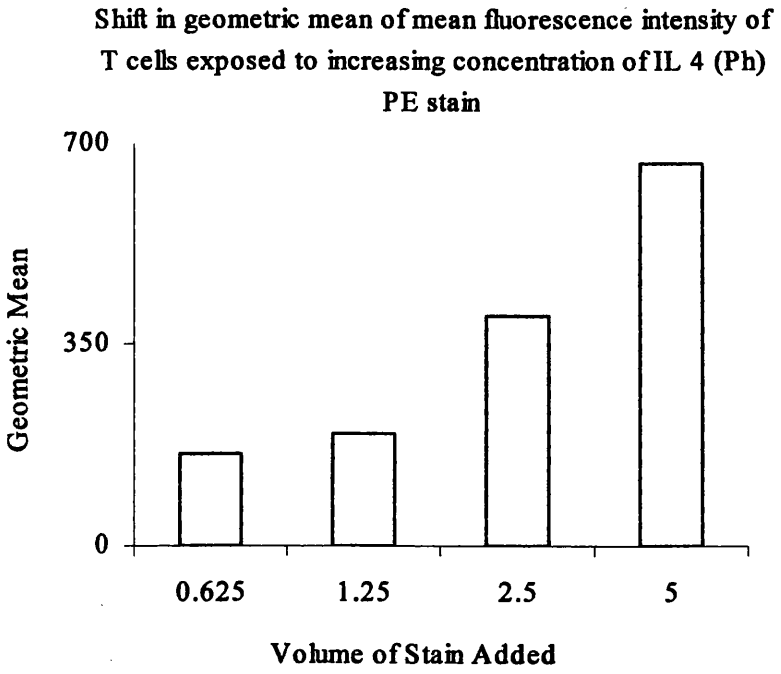


Figure 12-5 Relationship between volume of IL-4 PE added and geometric mean of Mean Fluorescence Intensity of T-cells

12.2.9. BAL T cell purification; Percoll, Ficoll-Hypaque, filtration, adhesion.

Flow cytometry of whole peripheral blood is an established and comparatively unproblematic technique. The same technique is less frequently performed on broncho-alveolar lavage cells. Cells from patients with TB had highly variable cellular pleomorphism, with different proportions of macrophages, monocytes and lymphocytes. This study was exclusively directed towards examining cytokine secretion of T-lymphocyte subgroups. Data from other cell groups was not sought. High percentages of other cell subsets, particularly macrophages, tended to arise in cell populations from diseased segments of lungs from patients with tuberculosis. While these were readily differentiated from lymphocytes by their size and granularity, where present in high percentages they made the collection of data from cell populations extremely slow; the gated cell population aimed for was 10,000 events. In cell populations where only 1% of cells were lymphocytes, this made data collection slow and profligate in terms of reagents used in the flow cytometer. Attempts were therefore made to purify T-cell populations without losing significant proportions of active lymphocytes.

Methods

Several methods of purification of lymphocytes were attempted.

1. Crude filtration of lavage specimens was performed through sterile gauze to remove visible debris.
2. Density separation of cell populations by Ficoll-Hypaque and Percoll.
3. Removal of macrophages by adherence to culture flasks.

Filtration of BAL cells through Gauze

To confirm that crude gauze filtration had no effect on cytokine production, an aliquot of lavage was left unfiltered and compared to a filtered specimen. Cells were then stimulated, stained and fixed as described.

Results

There was no difference in cytokine production after gauze filtration.

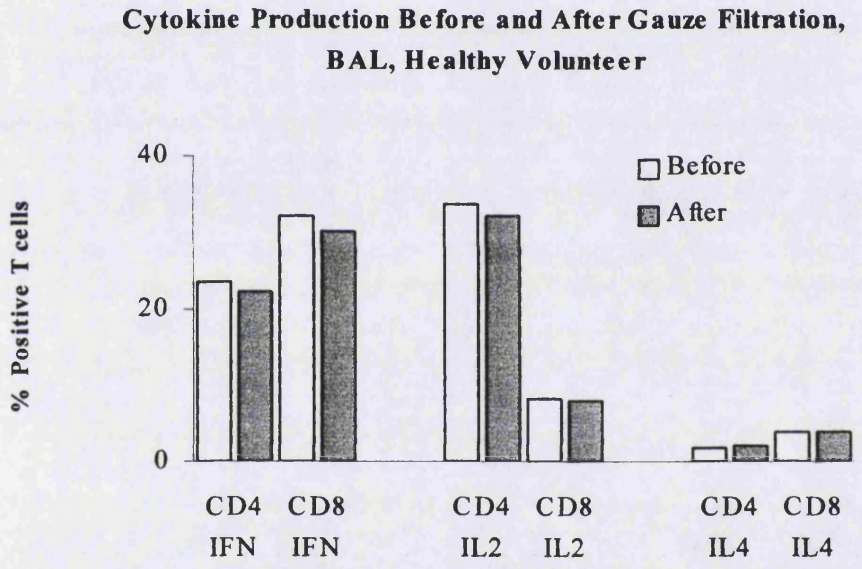


Figure 12-6 Effect of gauze filtration on cytokine production

Separation of lymphocytes by Ficoll-Hypaque

Before the whole blood method of preparation of peripheral blood mononuclear cells was perfected, Ficoll-Hypaque density separation of lymphocytes was an integral step in the process . The technique was attempted with BAL.

Cells were prepared in RPMI-1640 and 10% FCS as described. One 5ml aliquot of cells ($4x10^6$/ml) in RPMI was carefully layered onto 20ml sterile Ficoll-Hypaque in universal tubes and centrifuged at 17,000 rpm for 25 minutes (400g), brakes off. The Ficoll-Hypaque separated out into four layers according to density; an upper RPMI layer, an intermediate cellular layer, a layer of Ficoll-Hypaque and a sediment of red cells and debris. The layers were carefully aspirated by pipette and washed again in 5ml and then resuspended in 1ml RPMI + 10%FCS . 50μL of cells were collected from each layer, stained with Trypan blue equal volume, left for 5 minutes and counted for viable cells in a Neumann Haemocytometer, both for lymphocytes and other cells (macrophages and monocytes).

Results

Although some twofold concentration of lymphocytes was obtained into the intermediate cellular layer, there was unacceptable loss of viable cells, possibly in the wash step; the Ficoll-Hypaque did not adequately separate out lymphocytes into one specific layer (Table 12-2).

Layer	Percentage viable lymphocytes	Percentage other viable cells	Total number of viable cells/ ml
Unseparated cells	1.2	68.8	$4x10^6$
Upper layer (RPMI)	0	0	Nil
Intermediate layer (cellular buffy layer)	2.4	47.2	$0.5x10^6$
Ficoll layer	0	0	Nil
Lowest layer (debris/ rbc)	3.1	36.9	$0.65x10^6$

Table 12-2 Separation of cellular layers after Ficoll-Hypaque density separation

Discussion

Ficoll-Hypaque is conventionally used for separation of PBMCs from whole blood. The biophysical properties of lavage cells in RPMI and 10% FCS are plainly different, and Ficoll-Hypaque separation was therefore not considered appropriate as a means of lymphocyte purification.

Percoll Separation

Percoll is another commercially available means of gravity/ density separation of cell populations. Density of the gradient may be varied by diluting in PBS.

Methods

Lavage specimens were collected, from a patient with tuberculosis as described above. After washing and re-suspension in RPMI-1640 and 10% FCS the cells were counted into four

aliquots of 5ml 4 x 10^6/ml. One aliquot was set aside while the other three were carefully layered over 5ml Percoll 60%, 70% and 80% in 50ml universal containers. Percoll concentrations were derived from personal discussions with lab colleagues. The Percoll/ cell suspensions were then centrifuged at 1700 rpm for 25 minutes (400g). The Percoll separated out into layers: an upper layer of RPMI/ FCS (layer 1), one or two middle buffy cellular layers and a lower Percoll layer. These layers were carefully aspirated via pipette, washed and reconstituted in 1ml RPMI + 10% FCS. 100 µl was then collected, stained with CD3 monoclonal stain and fixed before being analysed by flow cytometry.

Results

Specimen	% Gated lymphocytes	% Gated cells staining CD3	Total cell number
Unseparated	1.22	91	61155
Percoll 60% layer 1	0	0	0
Percoll 60% layer 2	0.7	93	112350
Percoll 60% layer 3	1.82	67	165
Percoll 70% layer 1	0	0	0
Percoll 70% layer 2	0.63	96.1	36840
Percoll 70% layer 3	2.13	87.5	375
Percoll 70% layer 4	4.76	80	105
Percoll 80% layer 1	0	0	0
Percoll 80% layer 2	0.81	92.75	17070
Percoll 80% layer 3	0	0	0

Table 12-3 Results of separation of lavage cells by Percoll density gradient

Conclusions

None of the Percoll density gradients applied here adequately separated lymphocyte cell populations or increased the yield of lymphocytes in a way which resulted in sufficiently large concentrations of PBMCs to give meaningful results for flow cytometry. This method was therefore abandoned as a means of lymphocyte purification.

Increased concentration of lymphocytes by adhesion of macrophages to culture flasks; effect of delayed overnight storage on T-cell cytokine production.

A property of macrophages is that they adhere to the plastic of culture flasks (Nicod, Lipscomb et al. 1987). T lymphocytes do not share this property. The possibility that T-cells

might be harvested in higher concentrations by encouraging macrophages to adhere to culture flasks was therefore explored.

Clearly macrophages may act in a costimulatory capacity and affect T-cell cytokine secretion profiles. Although this was considered to be unlikely on the grounds that PMA stimulates already committed T-cells to produce previously encoded cytokines, it was clearly necessary to establish that cytokine production was not significantly affected either by delay or by extraction of macrophages.

Methods

Whole blood and BAL were collected from a healthy volunteer as described above. The blood and lavage cells were divided into two aliquots; one was stimulated immediately as described. The BAL specimen was stored at 35° in 5%CO_2 overnight in 500ml Falcon culture flasks. Blood was stored overnight at 4° C in heparinised glass bottles. Cells from the second aliquot were then stimulated identically to the first, for six hours. Specimens were then surface stained, lysed, permeabilised, stained with intracellular cytokines and fixed before flow cytometry. 5000 events gated by size and granularity to be lymphocytes were stored.

Results

Leaving BAL cells overnight in culture flasks resulted in a five-fold relative decrease in the number of cells required to yield 5000 lymphocytes (Table 12-4). These were confirmed to be lymphocytes by staining with CD3. Adherence did not appear to affect the eventual cytokine production of the T-cells (Figure 12-7). Nor did delay in stimulating the cells through overnight storage affect cytokine production.

	Total number of cells required to obtain 5000 lymphocytes	Percent lymphocytes (by gating)	Percent cells in lymphocyte gate staining CD3
Immediate	174216	2.87	96
Adhered overnight in culture flask	32680	15.3	94

Table 12-4 Altered percentage yield of lymphocytes after overnight incubation in culture flasks.

The reduction in cell numbers was due to reduction in numbers of macrophages (by gating); it did not appear to affect eventual cytokine production.

The production of cytokines with and without overnight delay is represented in Figure 12-7.

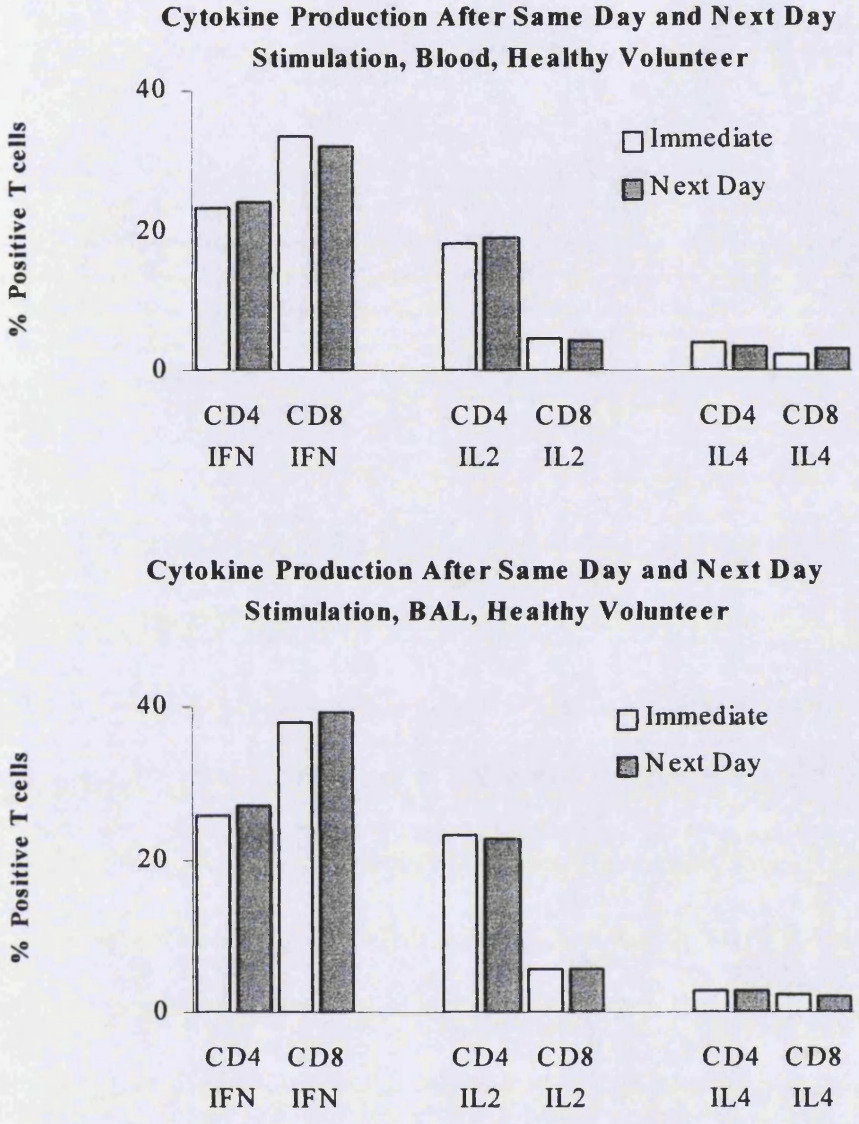


Figure 12-7 Effect of delay until following day on production of intracellular cytokines by T-cells

Discussion

Overnight storage did not appear to significantly affect the percentage of T-cells producing each cytokine, and incubation of BAL cells in culture flasks in RPMI-1640 + 10% FCS

decreased the number of macrophages which slowed FACS analysis and were not required for data interpretation. This was therefore adopted as standard protocol for these experiments.

12.3. Detection of intracellular cytokines using 3 colour FACS analysis of human peripheral blood mononuclear cells in whole blood, activated with PMA/calcium ionophore.

12.3.1. Methods

Cell culture and stimulation, whole blood

500μL of unstimulated cells were withheld for cell surface staining and as unstimulated, unstained controls. 1 ml of whole blood containing <1% mucous heparin was incubated with an equal volume of double strength PMA+ionomycin+monensin in RPMI-1640 to give their final previously optimised concentrations of 2.0μM ionomycin and 3.0μM monensin and 12.5ng/ml PMA respectively , and incubated for 6 hours in a 37°C incubator with 5%CO_2.

Controls included cells without any stimulants; stimulated unstained cells and isotype controls.

Staining of unstimulated blood cells

Uncultured cells were aliquotted into 100μl volumes in Falcon LP2 tubes, and 2μl of the following cell surface stains were added:

Stain	Manufacturer	Surface marker	Volume	Catalogue number
FitC	Dako	CD4	2µl	C7069
PE	Dako	CD8	2µl	R0806
Cy5	Dako	CD3	2µl	C7067

Table 12-5 Manufacturer and catalogue number of cell surface stains, with volumes used.

Cells were then incubated in BD lysing buffer 2ml 1 in 10 for ten minutes, centrifuged at 2000 rpm for 30 minutes, washed in 2ml washing buffer (PBS + 0.1% Azide + 1% BSA, see appendix A) and fixed in fixing buffer (1% paraformaldehyde, in distilled water, appendix A) 1ml, ready for flow cytometric analysis.

Staining of stimulated blood cells

Cultured blood and culture medium was aliquotted into 200µL volumes in Falcon LP2 tubes. Cell surface stains were added (see Table 12-6), and then incubated for 30 mins at 4°C.

Fluorochrome	Antibody	Manufacturer	Volume	Catalogue number
FitC	Cytokine			
PE	CD8	Dako	2µl	R0806
Cy5	CD4	Dako	2µl	C7069

Table 12-6 Cell surface stains for FitC cytokines, manufacturer, serial number and volume

Fluorochrome	Antibody	Manufacturer	Volume	Catalogue number
FitC	CD4	Dako	2µl	F0766
PE	Cytokine			
Cy5	CD8	Dako	2µl	C7079

Table 12-7 Cell surface stains for PE cytokines, manufacturer, serial number and volume added.

Each volume was then incubated at room temperature for 10 mins in 10% BD lysing buffer 1.5 ml. Cells were washed in washing buffer then permeabilised in 0.5ml 10% BD permeabilising buffer for 10 minutes at room temperature. Cells were washed again in washing buffer, and antibodies to the cytokines listed in Table 12-1were added to the permeabilised cells in previously optimised volumes.

Cells were washed in 2ml washing buffer (PBS + 0.1% Azide + 1% BSA, see appendix A), centrifuged and fixed in fixing buffer (1% paraformaldehyde, in distilled water, appendix A) 1ml, ready for flow cytometric analysis.

12.4. Detection of intracellular cytokines using 3 colour FACS analysis of human bronchoalveolar lavage cells, activated with PMA/calcium ionophore.

Collection of BAL fluid for flow cytometry

BAL was collected from subsegmental bronchi, as above. BAL yields <40% of infused volume of saline were discarded as unlikely to give appropriate cellular yields.

BAL was filtered through sterile gauze, as described, to remove larger pieces of debris. Lavage was centrifuged @ 2000 rpm/ 5mins, and supernatant stored for analysis of cortisol and cortisone (see Ch 10). Cells were washed once in RPMI/10% FCS, then resuspended in 10ml RPMI/10%FCS. Cells were incubated overnight at 37 degrees/ 5%CO2 in Falcon 500ml cell culture Flasks.

BAL cells were vortexed, and then counted in Neumann haemocytometer chamber with Trypan blue dye exclusion test for cell viability. Cells were centrifuged and resuspended to give cell concentration of $8X10^6$ /ml.

500μL of unstimulated cells were withheld for cell surface staining and as unstimulated, unstained controls.

Cell culture and stimulation, BAL

1 ml of BAL $8x10^6$/ ml was incubated with an equal volume of double strength PMA+ionomycin+monensin in RPMI-1640 to give their final previously optimised concentrations of 2.0μM ionomycin and 3.0μM monensin and 12.5ng/ml PMA respectively, and incubated for 6 hours in a 37°C incubator with 5%CO_2.

Controls included cells without any stimulants and isotype controls.

Cell surface markers were added to unstimulated cells as below.

Staining of unstimulated BAL cells.

Uncultured cells were aliquotted into 100μl volumes in Falcon LP2 tubes, and cell surface stains were added as per Table 12-5. Cells were then incubated in BD lysing buffer 2ml 1 in 10 for ten minutes, centrifuged at 2000 rpm for 30 minutes, washed in 2ml washing buffer

(PBS + 0.1% Azide + 1% BSA, see appendix A) and fixed in fixing buffer (1% paraformaldehyde, in distilled water, appendix A) 1ml, ready for flow cytometric analysis.

Staining of stimulated BAL cells

Cultured BAL cells and culture medium were aliquotted into 200μL volumes in Falcon LP2 tubes. Cell surface stains were added (see Table 12-6 and Table 12-7), and then incubated for 30 mins at 4°C

Each volume was then incubated at room temperature for 10 mins in 10% BD lysing buffer 1.5 ml. Cells were washed in washing buffer then permeabilised in 0.5ml BD permeabilising buffer for 10 minutes at room temperature. Cells were washed again in washing buffer, and antibodies to the following cytokines were added to the permeabilised cells in previously optimised volumes (Table 12-1).

Cells were washed in 2ml washing buffer (PBS + 0.1% Azide + 1% BSA, see appendix A), centrifuged and fixed in fixing buffer (1% paraformaldehyde, in distilled water, appendix A) 1ml, ready for flow cytometric analysis.

12.5. FACS analysis of BAL and Blood cells

The fixed samples were run on the FACScan within 24 hours. The FACS was operated using the CellQuest software. The machine was set to its optimum instrumentation settings (scatter detector and amplifier, threshold and compensation) using previously set parameters:

12.5.1. Scatter detector and amplifier

Threshold

This was set with the forward scatter at 100.

Compensation

The FL1, FL2 and FL3 channels were tested using single MAbs labelled with FITC, PE and Cy5 respectively, and the channels were compensated appropriately:

FL1-FL2	1.0%
FL2-FL1	30.0%
FL2-FL3	4.0%
FL3-FL-2	30.0%

Desired cell populations (lymphocytes) were loosely gated and information for 10000 gated cell events was stored

Data analysis

The data files were read using CellQuest reader software for Macintosh. A tight region (R1) was drawn around lymphocyte population (Figure 12-8). Other regions were drawn around CD8+ (R2) and CD4+ (R3) T cell populations. Dot-plots for the gated T cells were plotted

with both the unstimulated cell samples and isotype controls to separate cytokine +ve and -ve cells; and the % of CD4 or CD8 T cells with intracellular cytokines were calculated.

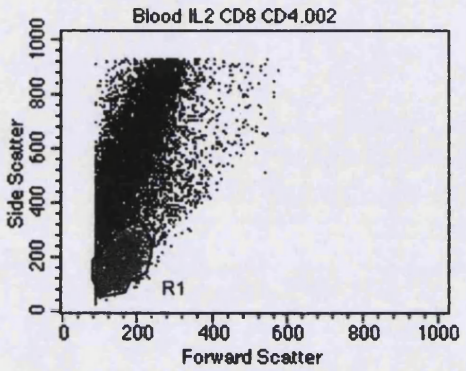


Figure 12-8. The R1 gate drawn around the lymphocyte poulation in FACS analysis of peripheral blood cells. A similar gate was used for lavage cells.

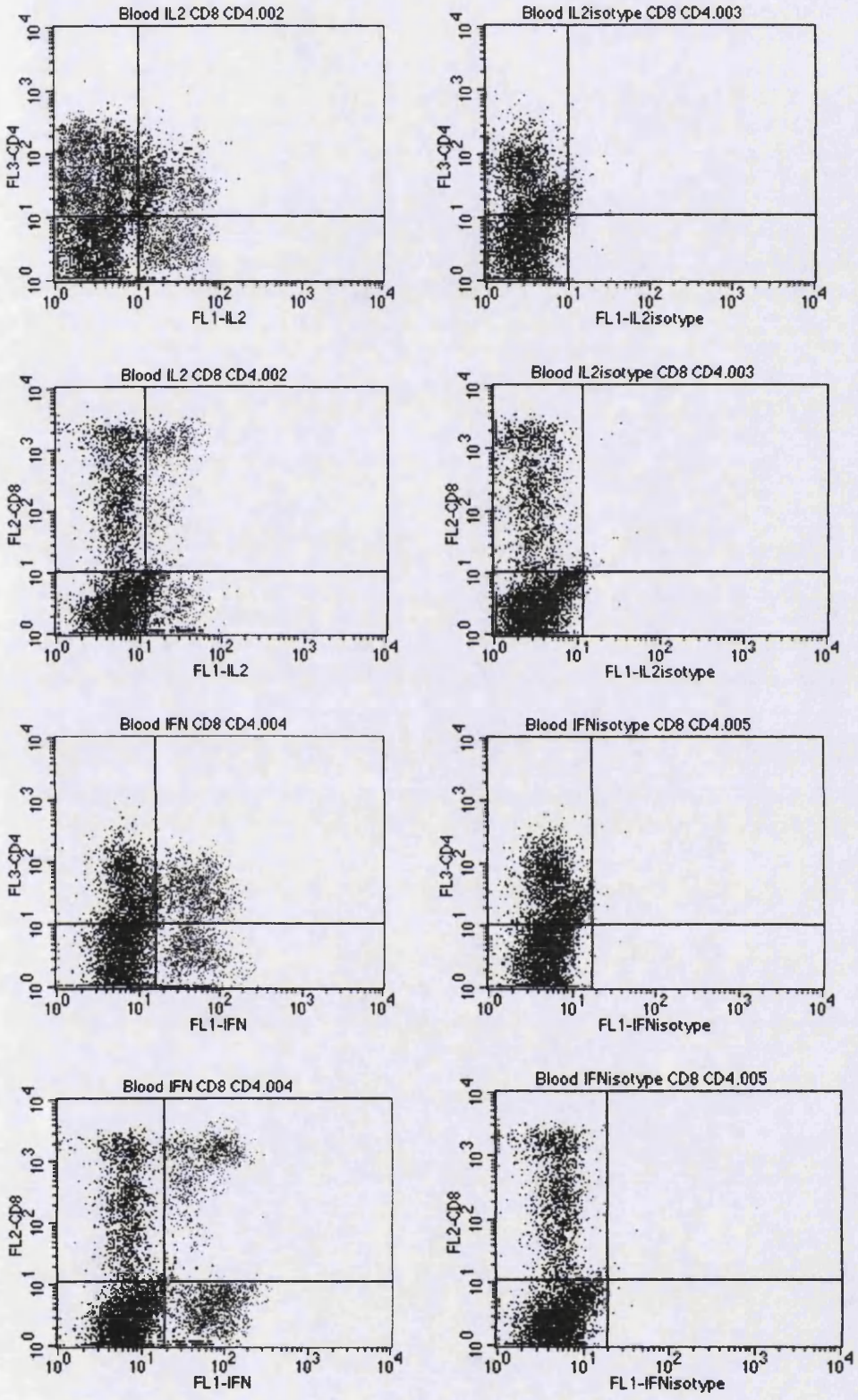


Figure 12-9. Flow cytometry dot plots of Th-1 cytokines and isotype controls from peripheral blood of a patient with pulmonary TB.

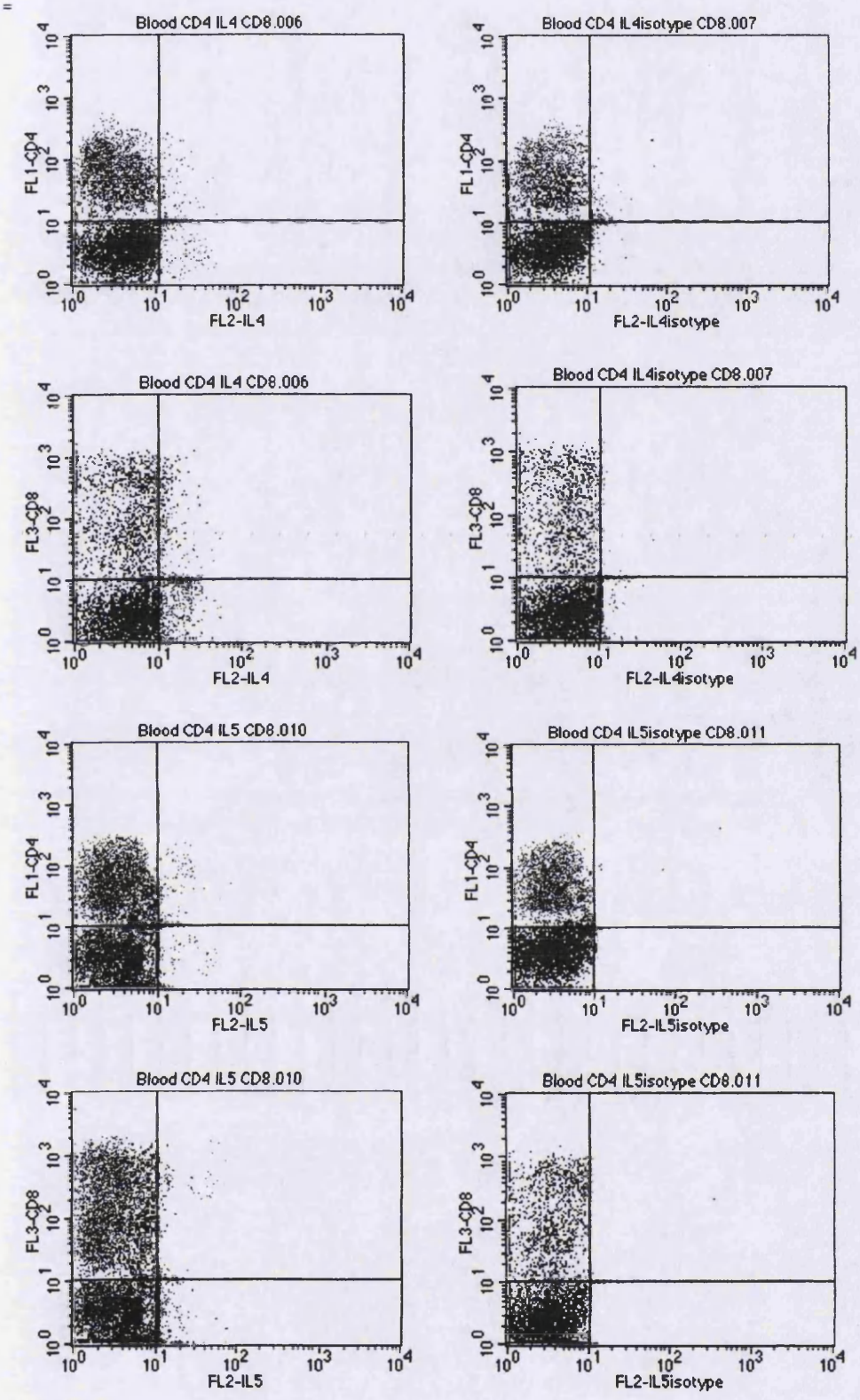


Figure 12-10. Flow cytometry dot plots of Th-2 cytokines and isotype controls from peripheral blood of a patient with pulmonary TB.

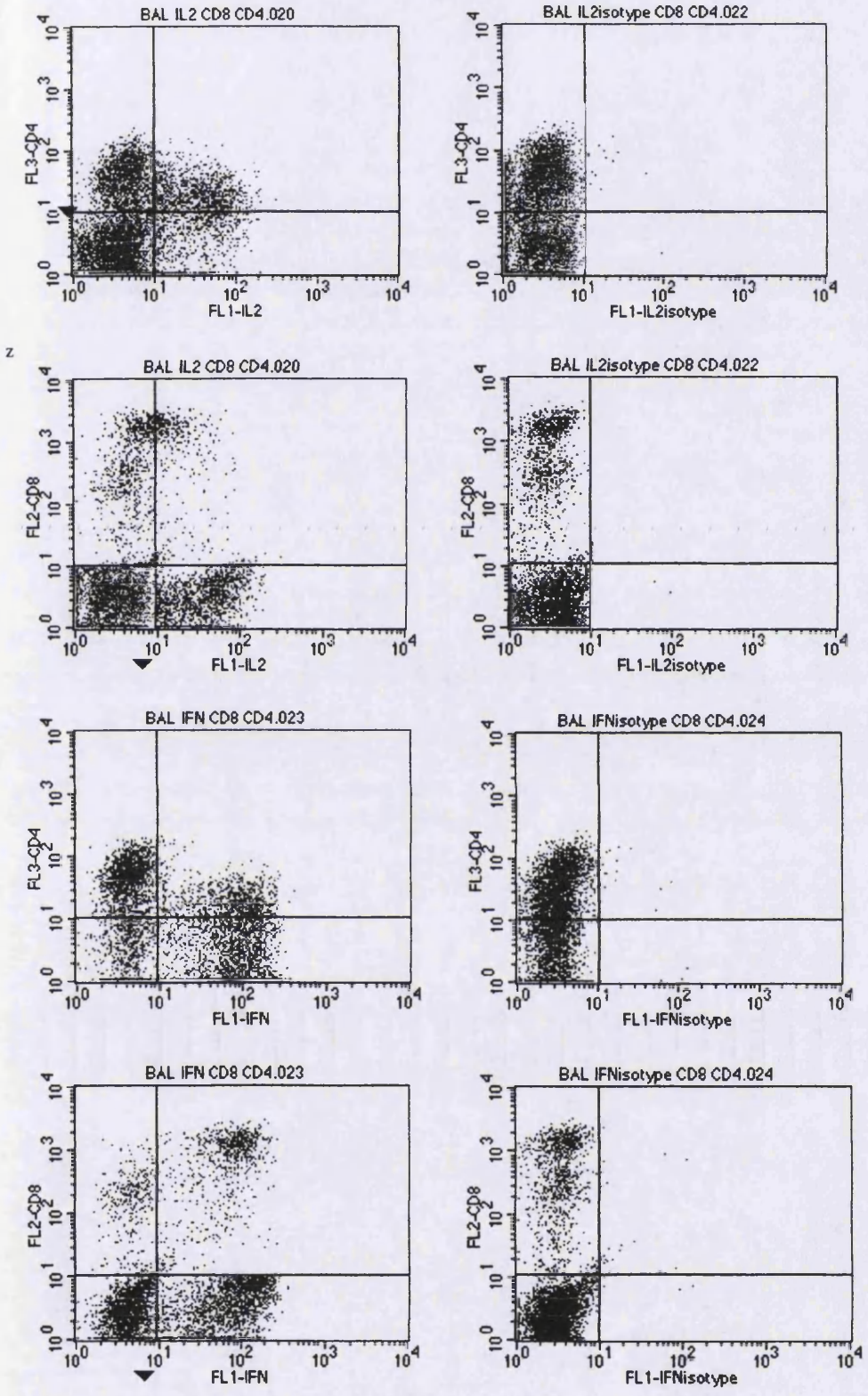


Figure 12-11. Flow cytometry dot plots of Th-1 cytokines and isotype controls from bronchoalveolar lavage of a patient with pulmonary TB.

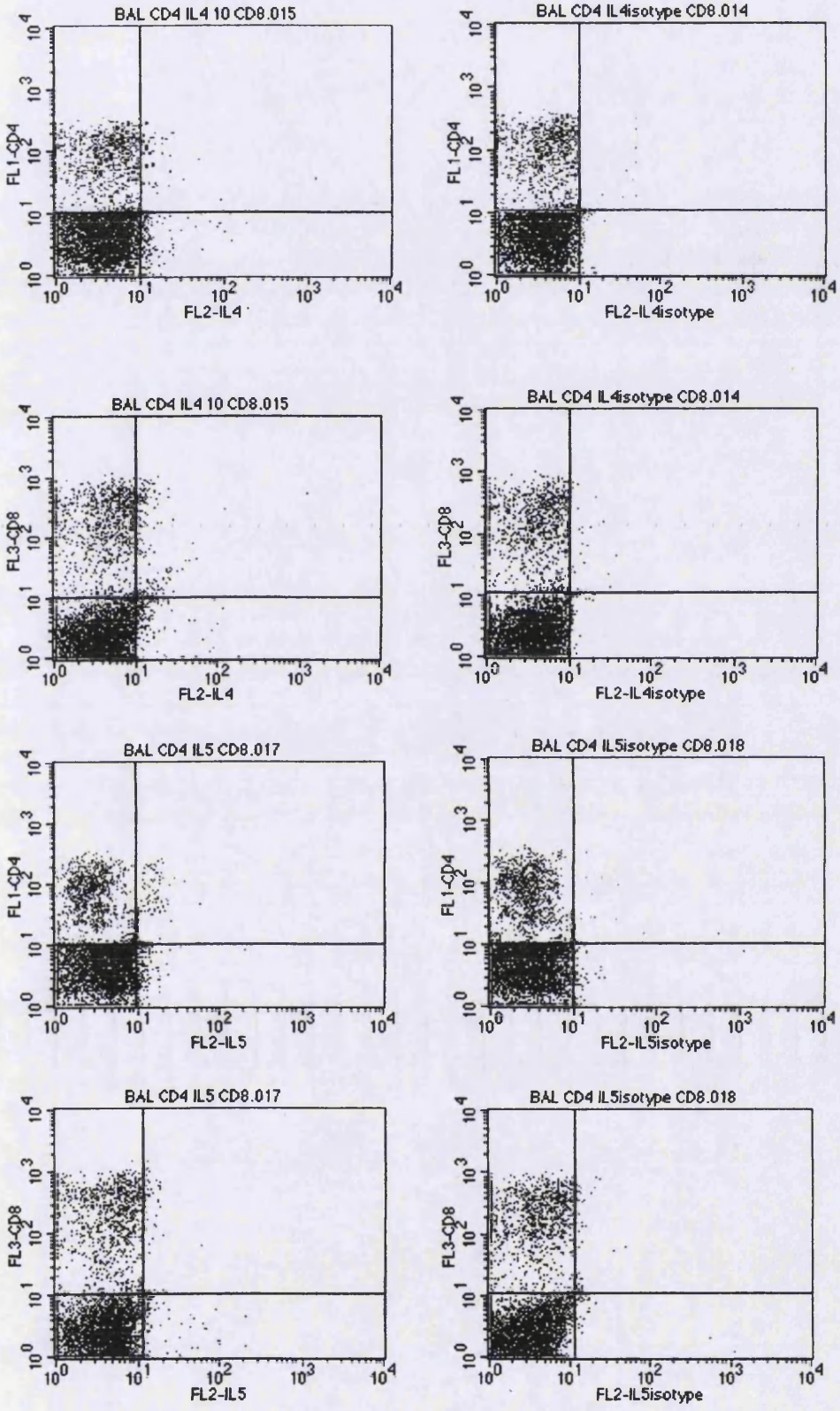


Figure 12-12. Flow cytometry dot plots of Th-2 cytokines and isotype controls from bronchoalveolar lavage of a patient with pulmonary TB.

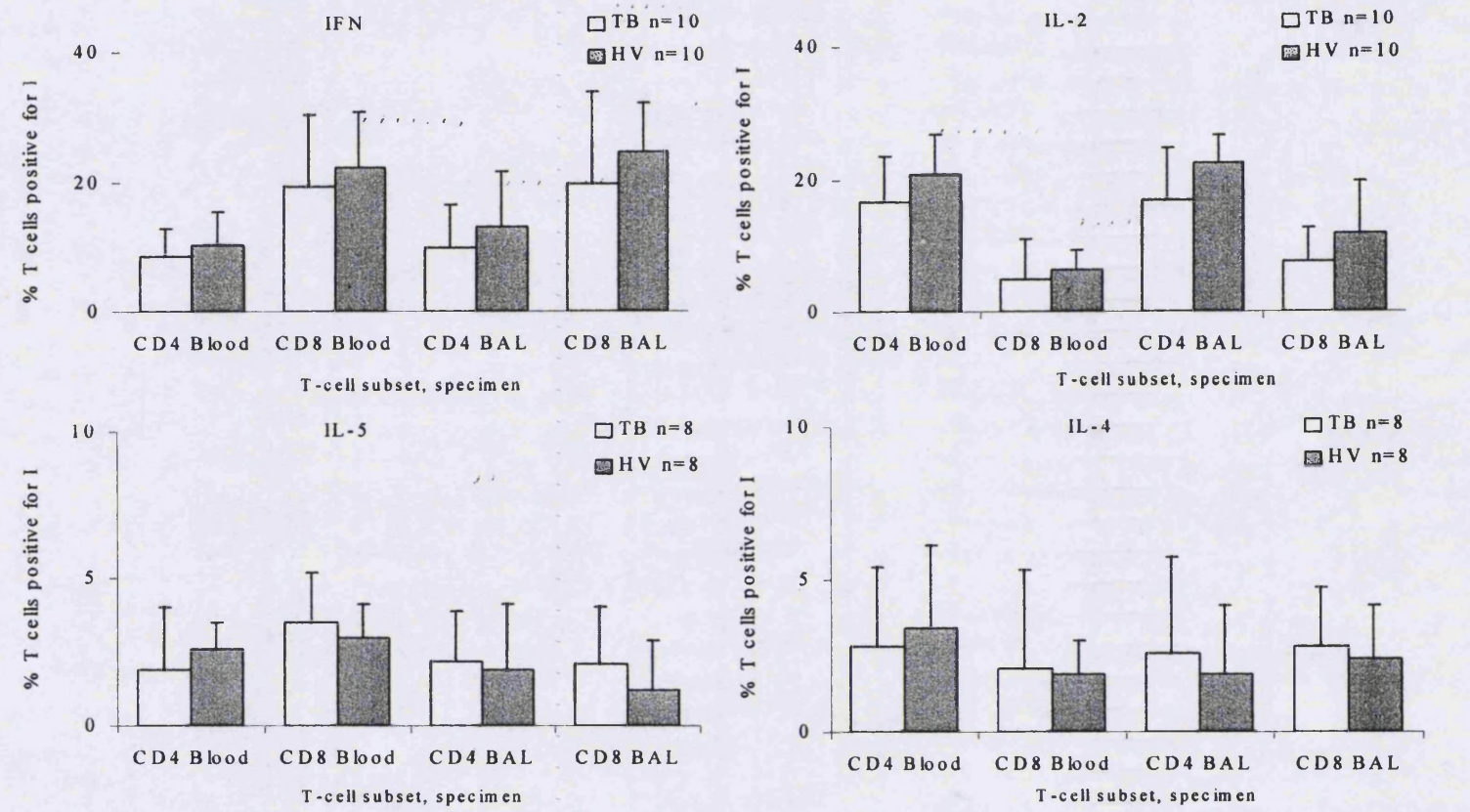


Figure 12-13 Percentage T-cells positive for Th1 and Th2 cytokines in blood and bronchoalveolar lavage.

12.6. Results

In these investigations, there were no significant differences between groups in number of T-cells secreting any particular cytokine. There was a trend for CD4 and CD8 cells in blood and bronchoalveolar lavage to secrete less IFN-γ and IL-2, however this was not significant. Neither was this significant when expressed as a ratio of CD4 IL-2/ IL-4, IFN/ IL-4 or CD8 IL-2/ IL-4 or IFN/IL-4 although predictably the trend was for ratios of Th1 to Th2 cytokines to be higher in the healthy volunteers.

The ratio of Th1 to Th2 cytokines in BAL did not correlate with cortisol to cortisone ratios in BAL. This was true of all Th1 and Th2 cytokines measured, whether CD4 or CD8.

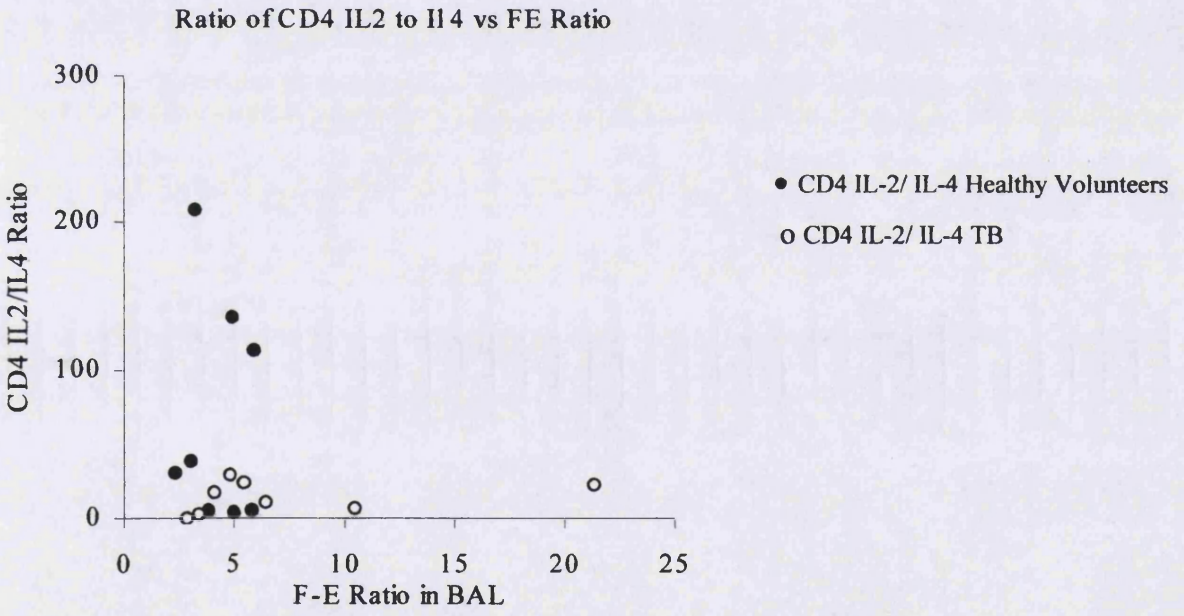


Figure 12-14 Ratio of CD4 IL-2/ IL-4 compared to cortisol to cortisone (F-E) ratio in BAL

12.7. Discussion

Flow cytometry is a powerful tool for rapidly analysing large numbers of cells for individual discrete parameters, such as the presence or absence of cell surface receptors or intracellular

cytokines. It makes comparatively crude estimates of the quantity of any molecule that would have been secreted into plasma in the absence of monensin. It is not particularly sensitive in detecting small numbers of cells secreting low concentrations of potent molecules. Interleukin-4 and Interleukin-5 plainly fall into this category. Low concentrations of these cytokines have potent biochemical effects and represent a Th2 T-cell response, as well as antagonising the Th1 response (Parronchi, De Carli et al. 1992). It is possible that T cells in blood and bronchoalveolar lavage were secreting increased quantities of Th2 cytokines, but the number of cells doing so was unchanged, and this increase was below the detection threshold of flow cytometry. At the percentages of cells involved, the number of T-cells secreting either cytokine could have been substantially increased without this being detected. Analysis of the number of copies of mRNA molecules for IL-4 and IL-13 by quantitative PCR would clarify this situation; these experiments are currently being performed within out institution and the preliminary results would seem to bear out this hypothesis (Seah, Scott et al. 2000).

The absence of a clear Th2 response in blood and BAL in no way negates the hypothesis. Mice with TB have been shown to alter their T cell repertoire with progress of the disease, being Th1 early in the disease, Th2 later and a mixed picture in the intermediate period (Hernandez-Pando, Orozco et al. 1996). These changes correlate with adrenal changes in size (Hernandez Pando, Orozco et al. 1995). Uninfected mouse adrenals hypertrophy early in disease, then atrophy to 50% of their pre-infection weight. The Th2 component tends to develop shortly before death of the animals, when they are clearly extremely ill. The patients recruited for this study would have been at varying stages in the progress of the disease; none had miliary TB and the severity of the illness could be classified as mild to moderate in all cases.

The lack of correlation between Th1/Th2 cytokine ratios in BAL and cortisol-cortisone ratios may reflect the possibility that other cytokines not measured in this study may have more effect on the cortisol-cortisone shuttle. It may reflect the small numbers with complete data (8 in each group). It should be noted however that there is an interesting spread of data in Figure 12-14. Those patients with TB who had a high cortisol to cortisone ratio had a low IL-2/IL-4 ratio. Healthy volunteers with a low cortisol to cortisone ratio showed a higher IL-2/IL-4 ratio. These groups appeared mutually exclusive. This was true of all Th1/ Th2 ratios measured. Although the numbers were small, it might be that this would be confirmed by larger sample sizes.

The only cytokines which have been clearly identified as influencing expression and activity of 11-β HSD 1 to date are TNFα and IL-1β (Escher, Galli et al. 1997). This study did not address production of these cytokines. The situation in clinical disease is probably highly complex, and involves cascades of interactions between cytokines. More in vivo and in vitro experiments are clearly justified in this area.

13.Activity of 11β hydroxysteroid dehydrogenase in mouse organs.

13.1. Aims/ introduction

The activity of 11-β HSD 1 in humans is influenced in direction and expression by a number of factors, including cytokines, particularly IL-1 beta and TNF alpha (Escher, Galli et al. 1997). Expression of these cytokines among others may be enhanced or suppressed by immunisation with Th1and Th2 inducing antigens such as *M. vaccae* and ovalbumin respectively. These cytokines are expressed in increased concentrations in tuberculosis in humans and mice (Law, Weiden et al. 1996; Orphanidou, Gaga et al. 1996).

This part of the study therefore sought to establish whether mice subjected to immune manipulation with pure Th1 and Th2 inducing antigens, both systemically and locally in the lungs, would demonstrate altered metabolism of tritiated substrate for 11-β HSD. In mice and rodents the predominant glucocorticoids are not cortisol or cortisone; but corticosterone and dehydrocorticosterone respectively. However the enzyme is not selective for substrate or product; mouse tissues metabolise cortisol and corticosterone identically.

13.2. Steroid extraction/ TLC

13.2.1. Optimisation

Extraction efficiency of steroids from tissue.

Livers were selected as having highest beta-counts for subjecting to serial elutions in ethyl acetate to establish the fraction of beta-counts remaining in tissues. Resuspended tissue tended to re-centrifuge poorly, increasing the possibility of organic tissue exposing substrate to further metabolism by active 11-β HSD from lysed cells. 11-β HSD is known to behave differently in lysed cells than intact tissue.

A snap-frozen liver from a balb/c mouse previously injected with 6 μCuries tritiated cortisol was ground, frozen, in a pestle and mortar on a bain-marie of dry ice and industrial methylated spirit; steroids were eluted using ice-cold ethyl acetate. Ethyl acetate supernatants were dried and reconstituted as for TLC below. Livers were subjected to three successive elutions.

Results

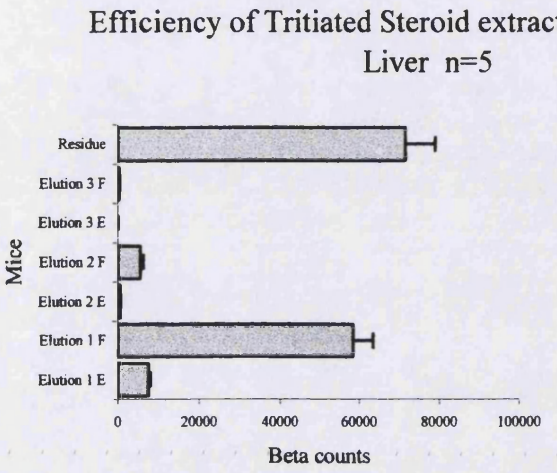


Figure 13-1 Efficiency of tritiated steroid extraction from liver in serial elutions

91% of available beta counts were extracted in the first elution, compared to 8.3% in the second and 0.7% in the third. The ratio of cortisol to cortisone was not significantly different between elutions (Figure 13-1). Overall extraction efficiency was 46%. The extraction efficiency for various organs is discussed below.

Conclusion

Extra elutions were considered time-consuming and likely to increase risk of active 11-β HSD metabolising available substrate in ethyl acetate, although this did not appear to be a problem in that ratios were comparable in successive elutions (Figure 13-3).

Background counts of TLC plates.

In order to document the effect of beta counts from TLC plates, scintillation fluid and background radiation, sections of TLC plate were cut and placed in scintillation fluid exactly as for specimens. The experiment was repeated using cold cortisol and cortisone on TLC

plates with an identical result. Counts were documented and the resulting average count (7cpm) was subtracted from all readings.

13.2.2. Thin Layer Chromatography; extraction of tissues and steroids

Snap frozen organs from animals injected with tritiated cortisol were ground, still frozen, in a pestle and mortar on a bain-marie of dry ice and industrial methylated spirit; steroids were eluted using ice-cold ethyl acetate to suppress the activity of extracellular enzymes. After centrifugation 20000 rpm for 30 mins at -8° the ethylacetate supernatant was dried overnight under a heated fan and reconstituted in 100 µl ethanol containing cold cortisol and cortisone standards. These were separated by TLC in 95% dichloromethane / methanol on aluminium TLC plates coated in fluorescent Silica. Spots of negative fluorescence under ultraviolet light corresponding to cold cortisol and cortisone were identified and cut out and placed in counting bottles containing scintillation fluid. Tritiated cortisol and cortisone were then read using a beta-scintillation counter; residual tissue from each organ was also placed in scintillation fluid to establish extraction efficiency.

Extraction efficiency of tritiated steroid from various organs

As mentioned above, extraction efficiency of tritiated steroid was assessed by measuring beta-counts in both eluted fluid and residual tissue, and calculating the proportion of counts in eluted fluid to total counts. The extraction efficiency varied greatly from organ to organ, according to size. Small organs like spleen and thyroid permitted up to 90% extraction efficiency, where in larger organs like liver it was reduced to 46%. This may have led to a source of error in assessing percentage conversion of cortisol to cortisone. Cortisol binds to cortisol binding globulin and other proteins in vivo and in vitro. The dynamics of cortisol and cortisone binding in vitro may well be different, with cortisol binding with greater affinity

than cortisone (Brian Walker, personal communication). This may have led to artificially higher cortisol to cortisone percentage conversion in larger organs like liver. It was not feasible within these experiments to assess cortisol binding. However the controls and subject mice had cortisol-cortisone conversion determined in identical ways. The object was to establish relative conversion of cortisol to cortisone in individual tissues after immunological challenge, rather than an absolute reference value. There was no linear correlation between organ mass and cortisol to cortisone conversion.

13.3. Protocol

Male Balb/c mice were immunised on day 0 and day 14 with *M. vaccae* 6μg per mouse, Ovalbumin 5μg on alum 100μg per mouse (see appendix A) or PBS. On day 28 groups of mice as specified in Table 13-1 were anaesthetised and challenged with intratracheal injections of M vaccae attached to nitrocellulose particles (see Appendix A), nitrocellulose particles alone or ovalbumin attached to nitrocellulose particles, or subjected to a sham procedure, according to Table 13-1. On days 31 and 34, 6 hours post challenge mice were anaesthetised (see appendix A) and sacrificed 30 minutes after injection of 10 μCuries of tritiated cortisol; blood was collected from the left ventricle immediately before sacrifice. Organs (lungs, thymi, livers, spleens and kidneys) were rapidly dissected, snap frozen in liquid nitrogen and stored at -70°C for subsequent steroid extraction.

Two mice died shortly after injection of intratracheal ovalbumin; one mouse was withdrawn as intratracheal injection was not considered to have been effectively administered.

Immunisation, Day=0 and 14	Challenge day=28	Sacrificed at Day 31	Sacrificed at Day 34		No of mice
M. vaccae	NC/MV	6	6		12
M. vaccae	NC	5	5		10
M. vaccae	Tracheotomy, no surgery		5		5
PBS	No surgery		7		7
				Total mice	32
Ovalbumin on alum	NC/OVA	5	5		10
Ovalbumin on alum	NC	5	5		10
Ovalbumin on alum	Tracheotomy, no surgery		5		5
	Dead	2			2
	Failed	1			1
				Total mice	28
				Overall total	**60**

Table 13-1 Immunisation/ challenge protocol

NC/MV = nitrocellulose particles attached to *M. vaccae* (See appendix A)

NC= nitrocellulose particles

NC/OVA = Nitrocellulose particles attached to Ovalbumin (See appendix A)

13.4. Results

The percentage conversion of tritiated cortisol to cortisone in mouse lung, liver and spleen is shown in Figure 13-2. Numbers for each group are identified in Table 13-1.

The percentage conversion of cortisol to cortisone varied considerably from organ to organ, reflecting the relative activities of GC 11β–dehydrogenases or reductases in each tissue (Figure 13-2 and Figure 13-3). This represents further evidence that each tissue regulates its local cortisol environment to suit its own needs.

The most striking observation occurred in the lung. In mice pre-immunised with ovalbumin and subsequently challenged with ovalbumin on nitrocellulose particles and sacrificed on day 31 (3 days post intratracheal challenge) there was a significant ($p<0.05$) increase in cortisone conversion within the lung compared to all other groups, including mice given PBS or *M. vaccae*. This effect appeared to have disappeared by day 34 (6 days post intrapulmonary challenge). Further, in the lungs, pre-immunisation with *M. vaccae* led to reduced conversion of cortisol to cortisone compared to controls given PBS alone.

The effects of *M. vaccae* (Th1) immunisation were similarly noted in the liver. In that organ there was less conversion to cortisone in mice immunised with *M. vaccae* and given an intrapulmonary challenge with nitrocellulose particles, on both days 3 and 6. This was significant only on day 6 ($p=0.02$). The effects were opposed by instillation of *M. vaccae* into the lungs.

The effects noted in the spleen showed similar trends to the lung, in that there was a tendency for ovalbumin to lead to more conversion to cortisone and *M. vaccae* to less. These effects were not statistically significant.

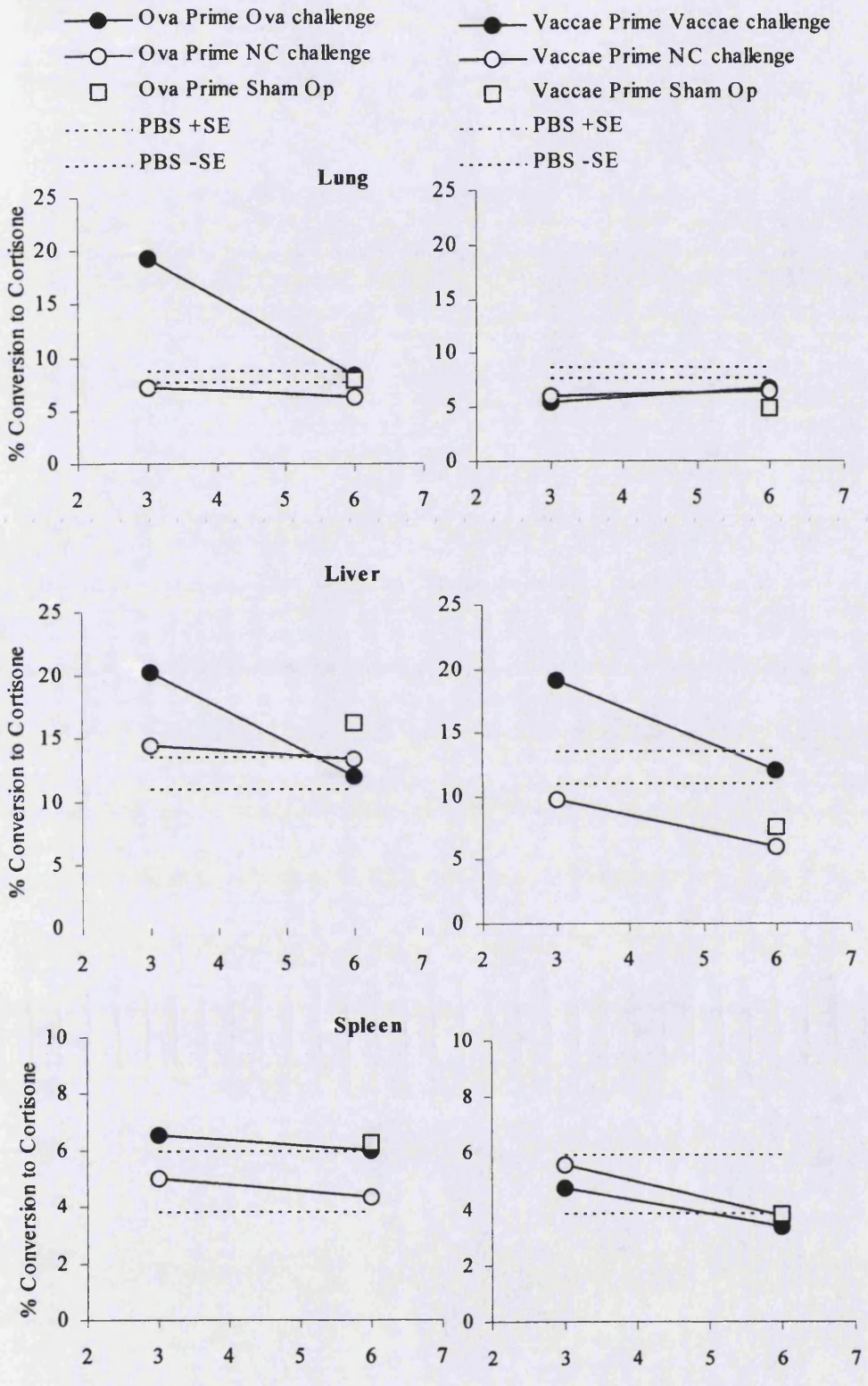


Figure 13-2 Conversion of tritiated cortisol to cortisone in lung, liver and spleen after intraperitoneal and intrapulmonary challenges. The x axis refers to days after intrapulmonary challenge, performed on day 28 of the experimental protocol.

Percent conversion of cortisol to cortisone in thymus, kidney and blood is demonstrated in Figure 13-3.

In thymus and kidney immunisation with *M. vaccae* led to reduced conversion of cortisol to cortisone, irrespective of pulmonary challenge. This was not statistically significant in thymus, and only significant in the kidney in the sham operated mice ($p=0.0002$). Ovalbumin immunisation, whether intraperitoneal or, intrapulmonary, had no significant effect on conversion. In blood neither *M. vaccae* nor ovalbumin had any effect on cortisol – cortisone conversion, either as immunisation or as pulmonary challenge. Blood cortisol and cortisone ratios are likely to represent the sum of activity of other organs.

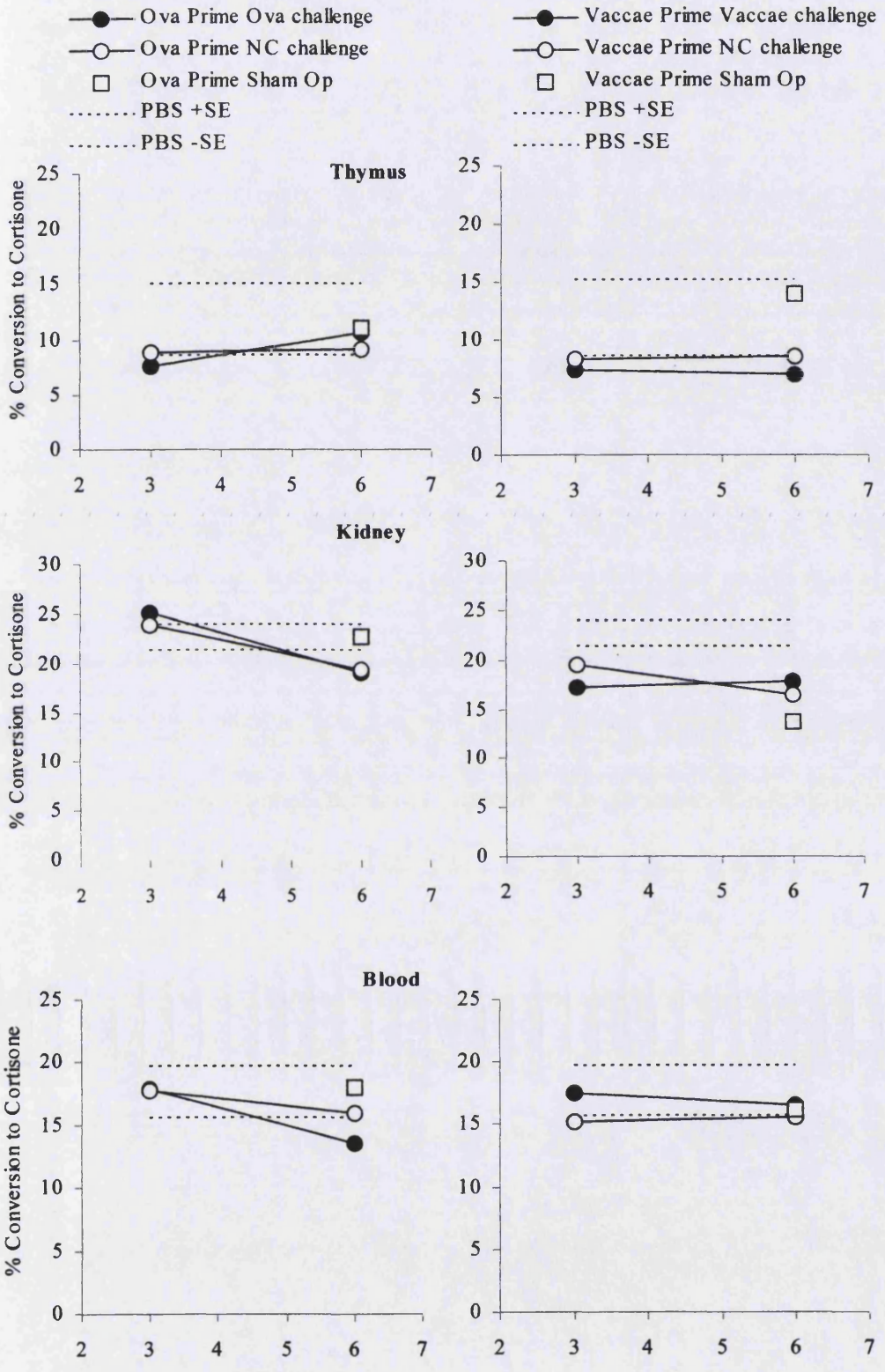


Figure 13-3 Percent conversion of tritiated cortisol to cortisone in mouse thymus, kidney and blood after intraperitoneal and intrapulmonary challenge. The x axis refers to days after intrapulmonary challenge, performed on day 28 of the experimental protocol.

In these experiments a complex series of variables was applied with different immunisation schedules and intratracheal challenges. While each organ seems to regulate its own conversion of cortisol to suit its own needs, there was clearly a complex time course involved as percentage conversion altered with time. In order to analyse the simplest effects of immunisation, the mice which were immunised and then subjected to a sham operation simulating intrapulmonary challenge but without injection of immune active agents were examined. The effects of immunisation alone on the cortisol-cortisone shuttle, as indicated by Figure 13-4, is striking. In this figure the conversion of tritiated cortisol in various organs in mice subjected to immunisation with Th1 or Th2 inducing antigens or PBS, and then a sham operation is compared. In the lung , liver and kidney immunisation with *M. vaccae* (Th1) resulted in significantly reduced conversion of tritiated cortisol to cortisone. The trend was also noted in the spleen, although this was not significant.

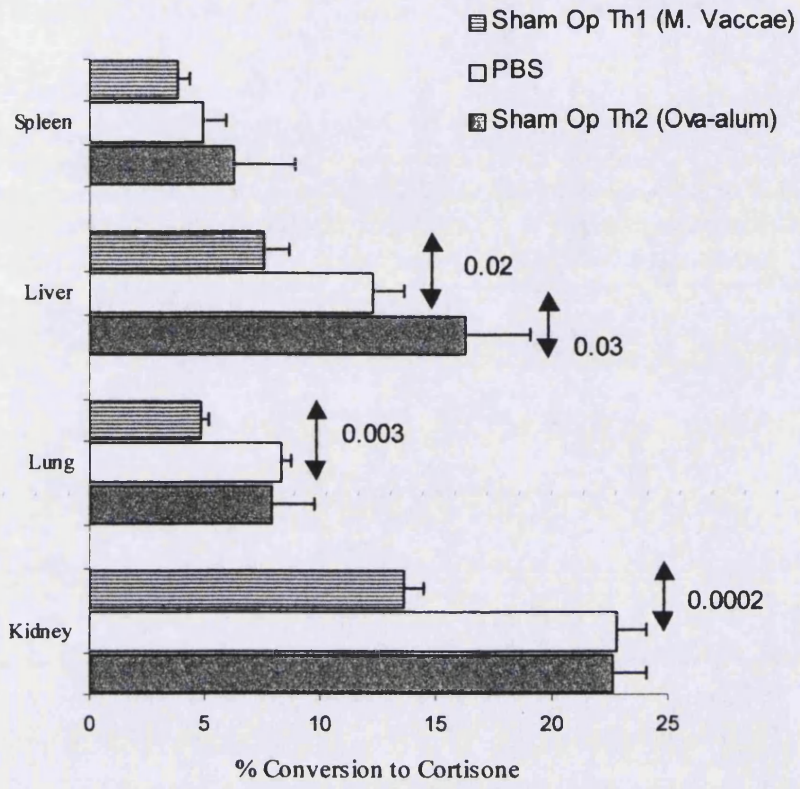


Figure 13-4 Effect of Th1-biased or Th2-biased vaccination on the equilibrium point between cortisol and cortisone in Balb/c mouse tissues, with significant p values (Mann Whitney U-test)

13.5. Discussion

The immunological response predicted from immunisation with ovalbumin, and subsequent instillation of ovalbumin into the lungs, would be Th2. These data provide further circumstantial evidence that dehydrogenase/ reductase activity in the lung is dependent upon immunological status, and that this occurs specifically in the lung, and that this effect as elicited by intratracheal ovalbumin and alum in the lung wears off after six days.

The effects of immunisation alone are particularly interesting (Figure 13-4). Immunisation with *M. vaccae* results in a Th1 pattern of immunity. In this experiment, those mice polarised to a Th1 response showed decreased conversion of cortisol to cortisone. This is similar to the situation observed in early tuberculous and BCG (Rook, Baker et al. 2000) and Figure 13-5) infection.

The effects of Th1 (*M. vaccae*) immunisation on the kidney are also interesting, as in this model there was reduced conversion of cortisol to cortisone. This observation is not contrary to the previous observation that cortisol is predominantly converted in the kidney to inactive cortisone. 11-β HSD 2, the enzyme responsible for the protection of the renal mineralocorticoid receptor described above, only functions as a dehydrogenase. However this activity is confined to the renal tubules. This experiment assessed overall cortisol-cortisone conversion in the whole kidney including the cortex and medulla, where other 11-β HSD enzymes are known to be active.

There is already some experimental evidence that 11-β HSD activity is affected by both cytokines and disease. Male balb/c mice were injected either with BCG $5x10^7$ or saline intravenously (Rook, Baker et al. 2000). Tritiated cortisol 10 μCi was injected 30 mins before sacrifice at serial time points. The lungs were dissected, snap-frozen, crushed and eluted steroids separated by TLC for beta counting. In early infection with BCG conversion of steroids within the lung favours cortisol, later in disease cortisone. These effects were not noted in the liver (Figure 13-5).These changes correspond to a shift from Th1 to Th2 type immunity, suggesting cytokine-mediated dysregulation of 11-β HSD 1, or increased expression of 11-β HSD 2.

In a similar experiment performed with a collaborating group in Mexico (Figure 13-6, Rook, Baker et al. 2000), mice were infected with *M. tuberculosis* and sacrificed at serial time points at days 3 to 90, and organs were handled as above. The same effects, but over a more prolonged time course, were observed. In early (Th1) disease, lung conversion favoured cortisol, later in disease (Th2) cortisone. Exactly the opposite effect was noted in the spleen. Liver and blood were unaffected.

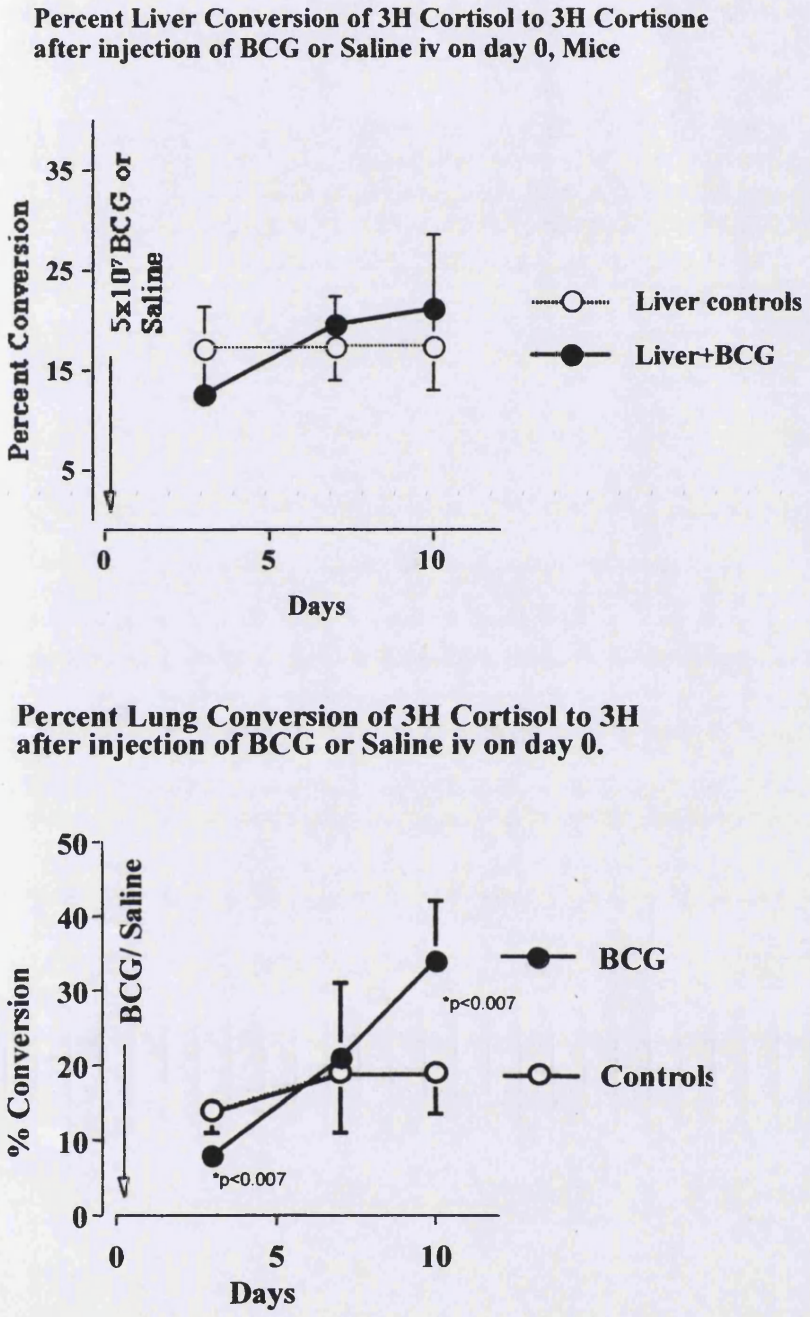


Figure 13-5. Conversion of cortisol to cortisone in mouse liver and lung, after injection with intravenous BCG (from (Rook, Baker et al. 2000).

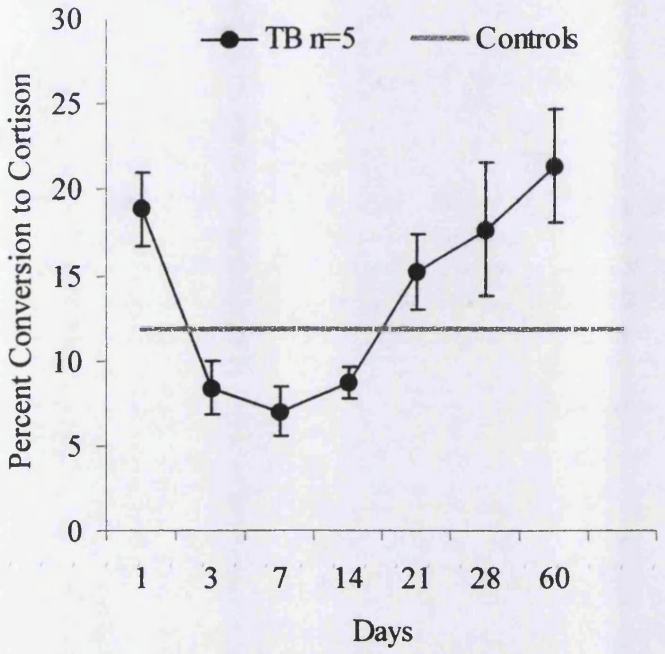


Figure 13-6 Conversion of tritiated cortisol to cortisone in tuberculous mice. (Rook, Baker et al. 2000).

There would appear from these experiments to be a complex time course of cytokine-mediated effects on the cortisol-cortisone shuttle in inflammatory conditions, at least in mice. It is interesting to note that the effects were observed in mouse lung even when the challenge was intravenous, and the liver was not affected as significantly (Figure 13-5).

13.6. Limitations of the study

While these data provide further evidence of the regulation of cortisol to cortisone conversion in various organs, they clearly have their limitations. As discussed above, a possible source of error arises from the variability of extraction efficiency from various organs. A further limitation arises from attempting to identify which enzyme responsible for conversion of

cortisol to cortisone is affected, 11 beta-hydroxysteroid dehydrogenase type 1 or type 2. Further enzymological studies and rt-PCR amplification of the relevant enzymes would elucidate these issues more clearly.

14. Regulation of 11 beta hydroxysteroid dehydrogenase by DHEA in mouse organs

14.1. Introduction

The effects of DHEA on glucocorticoid metabolism are outlined in the introduction. In essence, in humans at least it is an androgen precursor present in relatively high concentrations in plasma which antagonises the effects of glucocorticoids. In human tuberculosis in an earlier study DHEA output in urine collections was shown to be diminished (Rook, Honour et al. 1996). This finding was not confirmed in our study (Table 7-2); however overall output of any metabolite should be interpreted with caution where 24-hour collection was not controlled, and some of the patients were on anti-tuberculous medication. In rodents, the situation is different as plasma concentrations are much lower. However DHEA and DHEAS are plainly bioactive in rodents; probably via another analogue or metabolite.

There is a claim in the literature that dehydroepiandrosterone sulphate (DHEAS) can regulate the cortisol-cortisone shuttle (Homma, Onodera et al. 1998) However these authors used preposterous doses (10 mg/day for 70 days from six to 16 weeks of age), and the effects could be artefacts, or due to massive conversion to testosterone, or due to prolonged and massive peroxisome proliferation and liver damage. Doses of this magnitude also cause persistent priapism in male rodents.

In essence, they reported the effect of DHEAS on 11-β HSD activity in spontaneously hypertensive rats (SHR) (Homma, Onodera et al. 1998). SHR were given intraperitoneal injections of DHEAS as above. The dehydrocorticosterone/ corticosterone concentration ratio was significantly ($P< 0.05$) higher in the DHEAS group, suggesting that treatment with DHEAS enhanced the overall interconversion of corticosterone to dehydrocorticosterone. The activity of 11-β HSD in specific organs of the DHEAS group was affected, characteristic changes being increases in the kidney (14-58%), decreases in the liver (11-27%) and no change in the testis. Direct addition of DHEAS to 11-β HSD preparations from the kidneys of control SHR had the same effect as that observed in the in-vivo experiments. The fall in serum corticosterone in the DHEAS group was considered to be related, at least partly, to increased activity of kidney 11-β HSD.

The Homma paper is clearly uninterpretable, but the notion that there is no obvious receptor to explain the anti-glucocorticoid activity of DHEA, because it really operates via the shuttle enzymes, is an attractive one. In this study the effect of unsulphated DHEA in vivo on a group of normal male Balb/c mice has been further investigated .

14.2. Protocol

On day 0 and day 14 Male Balb/c mice were pre-immunised with *M. vaccae* in order to polarise their immune response to Th1 type. 11-β HSD 1 has been shown to be affected by cytokines, both in terms of direction and expression (Escher, Galli et al. 1997).

On days 28 and 29 DHEA 50μg was injected intraperitoneally in 100 μl olive oil suspension into one group (n=11) and olive oil alone into the second group (n=10). On day 29 mice were sacrificed 30 minutes after injection of tritiated cortisol 10 μCurie. Organs (livers, lungs and spleens) were snap frozen in liquid Nitrogen, and subsequently ground while frozen as described above for elution of organic steroids in ice cold ethyl-acetate. The steroids were then dried as above and reconstituted for thin layer chromatography.

Results (see Figure 14-1)

Mice given DHEA 50μg x2 showed a significant shift in the equilibrium point of the cortisol/cortisone shuttle, in favour of *cortisone* in the liver and lungs. Kidneys were unaffected. The effect was noted in the spleen but was not significant.

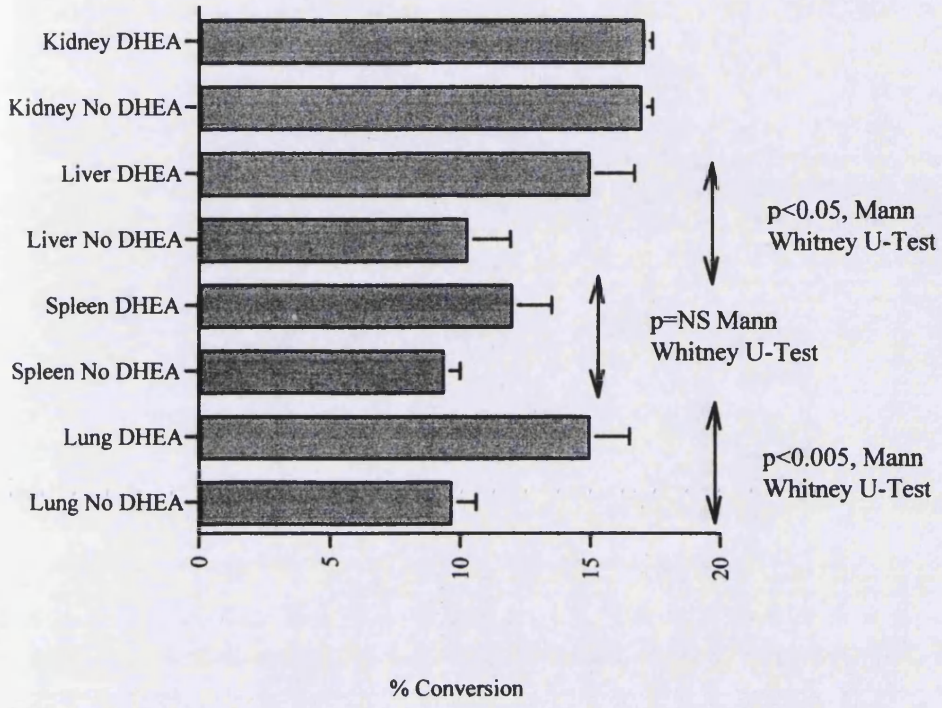


Figure 14-1 Conversion of tritiated cortisol to cortisone in mice with and without DHEA subcutaneously, pre-immunised with M. vaccae

14.3. Discussion

It should be noted that the effect demonstrated in the liver (Figure 14-1) was the opposite of that seen by Homma et al; this may be a dose related phenomenon as that group used DHEAS in concentrations of several orders of magnitude greater than in our experiment, likely to eventually damage the liver. Furthermore they investigated spontaneously hypertensive rats who are likely to have altered peripheral handling of glucocorticoids.

Whether the effect demonstrated in these investigations was due to enhanced dehydrogenase or blocked reductase activity in any organ was unclear, but it could explain the apparent anti-glucocorticoid activity of DHEA. This is a very attractive hypothesis because there is, at present, no established mechanism for the presumed action of DHEA, a molecule which is now believed to have a number of immunological functions. It would be enlightening to pursue the in vitro effects of DHEA on the cortisol-cortisone shuttle in a cell line, either liver or lung. This experiment needs to be repeated.

15. Discussion, Conclusions and Future Prospects

15.1. Discussion

Local cortisol concentration in any tissue is regulated by a reversible enzyme "shuttle" that can activate or inactivate cortisol by reversibly converting it to cortisone. This "shuttle", and the direction in which it operates (i.e. reactivating cortisone or inactivating cortisol) is regulated by numerous factors, including cytokines. Cortisol concentrations thus vary in sites of inflammation at different phases of an inflammatory response. Changes in local cortisol concentration can be largely independent of circulating cortisol levels.

Conversion of cortisol and cortisone was previously thought to occur principally in two sites, the kidney and the liver. In the kidney the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD-2) converts cortisol into inactive cortisone, and so stops cortisol from binding to the mineralocorticoid receptors (Walker 1994). This permits aldosterone to act as the principal mineralocorticoid. This is despite the fact that this hormone is present at much lower levels than cortisol, which has a high affinity for the receptor. Cells in the kidney that express mineralocorticoid receptors need to be protected from even physiological local cortisol concentrations to prevent their activation. By contrast the liver uses cortisol to regulate many enzymes, and to synergise with proinflammatory cytokines that trigger the acute phase response. Hepatic 11-β HSD type 1, a reversible oxidoreductase, rapidly converts cortisone back to active cortisol. Of key importance is the fact that the effective cortisol concentrations in the liver and kidney are totally different from each other, and both differ hugely from the values found in the serum, which is usually the only measure used clinically.

Is this local regulation of glucocorticoids important? One answer may be obtained by considering glucocorticoid activity in other tissues. Granulosa cells express 11-β HSD type 1 at some stages of the ovulatory cycle (luteinising) and so at that time may be sensitive both to cortisone (after conversion) and to cortisol, whereas at other times in the cycle (non-luteinised) the cells express only 11-β HSD type 2 and so will not be sensitive to either steroid (Tetsuka, Thomas et al. 1997).

Relative serum concentrations of cortisol to cortisone are a result of this conflicting "shuttling" between those steroids in different organs, together with the balance secreted by the adrenal. Circulating cortisol in normal individuals is about 5-10x higher than circulating cortisone. Analysis of the cortisol and cortisone metabolites appearing in 24 hour urine collections provide a more complete picture of the overall balance of cortisol to cortisone over a 24 hour period, using gas chromatography and mass spectrometry. (Rook, Honour et al. 1996).

Lysis of cells expressing 11βHSD-1 alters the balance of dehydrogenase to reductase activity. In intact liver cells, or transfected COS-1 cells, 11-β HSD type 1 is a reductase, but when these cells are homogenised the enzyme also behaves as a dehydrogenase. Conventional enzymological studies with tissue lysates do not reflect the in vivo situation. In order to investigate the equilibrium balance of cortisol to cortisone (i.e. the equilibrium point of the shuttle enzymes) in any one organ, it is therefore necessary to perform semi-in vivo experiments, or to use short-term organ cultures. A straightforward means of circumventing this problem has been described in chapter 13. Intraperitoneal injection of tritiated cortisol or cortisone, followed by extraction of steroids from the organs approximately 30 minutes later, gives accurate estimates of cortisol-cortisone ratios. A similar method (using prednisolone rather than a radiolabelled steroid) has been successfully used to show the dominance of dehydrogenation in kidney, and reduction in liver in vivo (Escher, Frey et al. 1994).

In tuberculosis patients the ratio of metabolites of cortisol to metabolites of cortisone is heavily biased towards cortisol in 24 hour urine collections (Rook, Honour et al. 1996). If this shift were due to failure of conversion to cortisone in the kidney, it would lead to hypertension and salt retention, the opposite of what is observed in tuberculosis. When cortisol concentrations were estimated after oral intake of cortisone (Chapter 8) they demonstrate that it is in fact due to accelerated "single pass" conversion of cortisone to cortisol. The site of this disturbance might be the liver, but the lung presents an attractive alternative. In the lung the predominant enzyme is 11-β HSD type 1, resembling the liver. It might be expected to behave as a reductase, though one published report suggests that in the lungs of normal donors the interconversion is running in reverse, with net transformation of cortisol into inactive cortisone (Hubbard, Bickel et al. 1994). This suggests two possible explanations.

The first is that 11-β HSD–1 is a reversible and regulated oxidoreductase, that happens to be functioning as a dehydrogenase (oxidase) in lung. This hypothesis is supported by the observation that in cultured hepatocytes the enzyme functions as a reductase, whereas in leydig cells it is primarily oxidative (Gao, Ge et al. 1997). The second is that the net conversion to cortisone is in fact due to the presence in lung of the other enzyme, 11-β HSD type 2, as has been reported (Suzuki, Sasano et al. 1998). The type 2 enzyme is also present in endothelial cells of the aortic arch of rats. It may be reasonable to suppose that it is also present in the vasculature of the lungs (Brem, Bina et al. 1998).

Either of two hypotheses - that Th1-mediated inflammatory response to mycobacteria in the lung may either lead to a change in the function of the type 1 enzyme, or a change in the

relative activity of the type 1 and type 2 enzymes – would fit the observed data. Work with a cell line in which the enzyme is normally bidirectional supports the former hypothesis. When cells were exposed to IL-1 or to TNFα , there was an increase in mRNA encoding 11-β HSD type 1 and in the quantity of enzyme produced. There was also an increase in the activity of the enzyme, but this was exclusively reductase (Escher, Galli et al. 1997). If the same situation applies in human tuberculous lung, it will start to contribute to the systemic pool of cortisol at the expense of circulating cortisone. This would provide an explanation for the findings in urine collections, and perhaps cause perturbation of the local immune response (Rook, Honour et al. 1996).

Experimental support for this hypothesis has already been described. (Figure 13-5). There are striking swings in the balance of cortisol to cortisone in different organs following intravenous immunological challenge. Under normal circumstances in mouse lung the equilibrium point is about 15% cortisone. Three days after i.v. BCG, during the early phase of TNFα, IL-1 and Th1 cytokine production, the lungs of Balb/c mice fail to inactivate cortisol to cortisone. Subsequently, by 10 days, when there is a large Th2 component, there is an impressive swing to inactivation. Much of the injected cortisol then appears in the lungs as cortisone (Figure 13-5). Similar findings have emerged from a study of murine pulmonary tuberculosis, where again the cortisol/cortisone equilibrium in the lungs shows a switch to cortisol during the Th1-dominated phase, followed by a switch to cortisone in the later Th2 phase (Figure 13-6).

In fact the shuttle enzymes are subject to complex regulatory control, and in addition to IL-1β and TNFα (Escher, Galli et al. 1997) and steroid hormones (Voice, Seckl et al. 1996;

Waddell, Benediktsson et al. 1996) they are also regulated by thyroid hormone and growth hormone (Gelding, Taylor et al. 1998).

Ultimately cortisol appears to affect lymphocytes in much the same manner as the cortisol-mediated effect of stress (Zwilling 1992; Brown, Sheridan et al. 1993; Bernton, Hoover et al. 1995). It seems to to drive the immune response towards a Th2 cytokine profile (Fischer and Konig 1991; Wu, Sarfati et al. 1991; Guida, O'Hehir et al. 1994; Padgett, Sheridan et al. 1995; Hernandez-Pando, de la Luz Streber et al. 1998). This applies in vitro as long as appropriate concentrations of glucocorticoid are used. However naive and memory T cells are affected differently (Brinkman and Kristofic 1995). Newly responding naive T cells are deviated towards Th2, despite the fact that IFNγ secretion by memory T cells is rather resistant to glucocorticoids (Brinkman and Kristofic 1995). Whole peripheral blood mononuclear cell populations stimulated with PHA and then exposed to glucocorticoids demonstrate that IL-5 production is more easily suppressed than IL-2 or IFNγ (Van-Wauwe, Aerts et al. 1995) This persistence of Th1 cytokines is attributable to memory cells (Brinkman and Kristofic 1995). A similar situation was noted when T cell clones were grown from bronchoalveolar lavage (BAL) samples using PHA and IL-2 as the stimulus. Here dexamethasone (at supraphysiologic doses) inhibited anti-CD3-induced production of IL-4 and IL-5 more than it inhibited production of IFNγ (Krouwels, van der Heijden et al. 1996). BAL T cells are known to be activated memory cells, so this is compatible with the results of Brinkmann et al. (Brinkman and Kristofic 1995). It does not alter the fact that if an immune response in naive cells is allowed to develop in the presence of cortisol or cortisol analogues, a Th2 line will develop. Ramirez et al have demonstrated this phenomenon using spleen cells from "clean" laboratory rodents (Ramirez, Fowell et al. 1996) which have few memory cells under normal circumstances. Over time therefore raised physiological concentrations of glucocorticoid will

deviate the response to Th2. This may be because cortisol stops IL-12 from being secreted by antigen presenting cells (APCs) and makes them secrete IL-10 instead (Vieira, Kalinski et al. 1998; Visser, van Boxel et al. 1998). Raised glucocorticoid concentrations might arise due to systemic increases, or to local rises due to shifts in the shuttle equilibrium, as explained above.

Glucocorticoids severely compromise the ability of human and murine macrophages to control the growth of *M. tuberculosis* in vitro (Rook, Steele et al. 1987). Restraint stress in mice enhances mycobacterial growth in vivo and this has been shown to be directly attributable to glucocorticoids (Brown, LaFuse et al. 1995). The ability of GC to enhance mycobacterial growth in murine macrophages in vitro is only seen using macrophages from Bcg s mice and not from Bcg r mice (Brown, LaFuse et al. 1995). Bcg s and Bcg r are alleles of an autosomal dominant gene, Nramp1, which affects innate resistance of mice to mycobacteria and to several other intracellular pathogens. However levels of cortisol will certainly influence local release of the proinflammatory cytokines, IL-1 and TNFα (Joyce, Steer et al. 1997, Zhang, Zhang et al. 1997).

15.2. Conclusions from this thesis

This thesis describes how local regulation of effective cortisol concentrations is largely independent of circulating cortisol concentrations, and is itself regulated by local cytokine production. It also outlines the ways in which cortisol affects the function of macrophages and T cells that are most relevant to mycobacterial disease. It represents the most complete analysis of glucocorticoid metabolism in tuberculosis ever performed. This work is important for several reasons.

1) It provides insight into new target molecules for the treatment of tuberculosis, which is a rapidly increasing global emergency. Murine work, based on this study, has already shown promise in this respect.

2) It may explain the paradoxical failure of Th1-mediated immunity in TB. As described above, TB patients have failing Th1 responses, and increased IL-10 and TGFβ (Ellner 1997). Locally increased cortisol in TB lesions can explain this, so this work unravels the next stage of the conundrum.

3) It provides the first ever *in vivo* demonstration of changes in peripheral regulation of cortisol resulting from an inflammatory process; and in a highly important clinical model. Previous work has only demonstrated this phenomenon *in vitro* in a cell line (Escher, Galli et al. 1997). The observations have been confirmed in a mouse model both in tuberculosis and other inflammatory models. The concept of local regulation of inflammation by the so-called cortisol-cortisone shuttle has implications for all inflammatory lesions, not just tuberculosis.

4) It has suggested, for the first time, a possible mechanism for the poorly understood mechanism of anti-glucocorticoid action of DHEA, operating through the cortisol-cortisone shuttle and enhancing the conversion of cortisol to inactive cortisone.

5) The observations made here may not be confined to tuberculosis, but may be important in other mycobacterial disease such as leprosy (Rook and Baker 1999). Shuttle enzymes are present in skin, and the abrupt reversal reactions which characterise progression of active leprosy may be related to cytokine mediated alterations in local glucocorticoid metabolism.

This study has not addressed the mechanism for altered cortisol metabolism in tuberculosis but possibilities include dysregulation of 11-β HSD 1 by cytokines including IL1β and TNFα (Escher, Galli et al. 1997). One or both of these cytokines is present in increased concentrations in bronchoalveolar lavage fluid and pleural effusions in human (Law, Weiden et al. 1996; Orphanidou, Gaga et al. 1996) 1and murine tuberculosis (Hernandez Pando, Orozco et al. 1997). This hypothesis is further supported by murine models of tuberculosis in which we have shown shifts in 11-β HSD activities that correlate with patterns of cytokine release (Figure 13-5 and Figure 13-6). Since cytokines will be normalised by treatment of infection, this mechanism also provides a reasonable explanation for normal cortisol metabolism observed in patients cured of tuberculosis.

15.3. Possible future prospects from this thesis.

It is clear that there are other investigations of the so-called cortisol-cortisone shuttle that could be pursued as a consequence of these investigations. The genes coding for both 11-β HSD 1 and 11-β HSD 2 have been identified and cloned in mice (Rajan, Chapman et al. 1995). It would clearly be theoretically possible to quantify mRNA expression of this enzyme in the lungs of mice with tuberculosis and other inflammatory models, and thereby prove that regulation of expression of the enzyme is altered in these circumstances. The observation that DHEA affects the shuttle could also be pursued in an in vitro model.

Whether or not the shift in cortisol metabolism in favour of the active glucocorticoid is a primary or secondary phenomenon in reactivated pulmonary tuberculosis, these observations have important therapeutic potential. Manipulation of corticosteroids such as corticosterone

and androstenediol have been shown to affect survival curves in a mouse model (Hernandez-Pando, de la Luz Streber et al. 1998). It may be that inhibitors of the reductase activity of 11-β HSD 1 administered to patients with tuberculosis would enhance their ability to mount a normal hypothalamo-pituitary-adrenal axis response to infection, but lower the basal concentrations of cortisol in key target organs particularly the lung. The interactions between the immune system and the factors controlling glucocorticoid secretion and sensitivity therefore supply a new approach to tackling the expanding problem of tuberculous infection.

16. Appendix A. Reagents and buffers.

Washing buffer for flow cytometry

PBS + 0.1% Azide + 1% BSA. Make 500ml, store at 4^0 for maximum 2 months

Fixing buffer for flow cytometry

1% paraformaldehyde, in distilled water. Make 500ml, store at 4^0 for maximum 6 months

Lysing Buffer

Becton Dickinson catalogue number 349202. Dilute 10% in washing buffer immediately before use.

Permeabilising Buffer

Becton Dickinson catalogue number 340457. Dilute 10% in washing buffer immediately before use.

Carbonate buffer

1.6G Na_2CO_3 (15mM)

2.9G $NaHCO_3$ (35mM)

0.2G NaN_3 (3.1mM)

H_2O to 1 litre

Adjust to pH 9.5

Ovalbumin solution

Deionised water or PBS

2mg/ml ovalbumin (Sigma) in sterile deionised water/ PBS

Filtered through sterile 0.2μm filter

Read solution on A280 spectrophotometer (against water or PBS standard)

Dilute to 1mg/ml, read again on spectrophotometer

Ovalbumin on Alum

Aiming for 5μg ovalbumin and 100μg alum per mouse

1. 1ml 10% AlK $(SO_4)_{12}$ H_20
2. Add 2.28 ml 0.25M NaOH while vortexing
3. Incubate 10 mins at room temperature
4. Centrifuge for 10 mins at 1000g
5. Discard supernatant and add 5ml dH_2O to pellet to redissolve
6. Add 500μl 1mg/ml ovalbumin solution
7. Mix and let incubate for 20 mins room temp
8. Spin 1000g 10 minutes
9. Discard supernatant, resuspend pellet in 10ml N Saline

Nitrocellulose Particles + *M. vaccae*/ ovalbumin

1. Pipette 5μg ovalbumin solution or 6μg sonicated *M. vaccae* 1mg/ml per 20mm2 nitrocellulose sheets, supported on sterile needles. Use plain nitrocellulose as controls

2. Dry sheet for 90 mins

3. Dissolve sheets in 133μl DMSO per 20 mm^2 nitrocellulose

4. Rotate for 90 minutes to dissolve fully

5. Add 3x volume of carbonate buffer drop by drop while vortexing to precipitate particles

6. Allow to stand for 5 minutes to precipitate larger particles

7. Pipette solution from larger particles

8. Centrifuge 1500rpm 10 minutes, discard supernatant

9. Resuspend in 5ml carbonate buffer

10. Rinse once in sterile deionised water, centrifuge 1500 rpm 10 minutes, discard supernatant

11. Resuspend in 1.5ml deionised water in eppendorf

12. Centrifuge in quickspin 15 minutes at max speed

13. Resuspend in in 0.9% saline 625μl

14. Use 50μl per intratracheal wash

17.Appendix B. Anaesthetic agents for rodents

Parenteral anaesthesia for rodents

Hypnorm dilute 1/1 in water

Midazolam dilute 1/1 in water

Mix together (= Rodent Anaesthetic Mixture; RAM)

3 ml/kg i.p.- a 30 gm mouse gets 0.1 ml i.p.

18.Index

19.References

Agarwal, A. K., Monder, C., Eckstein, B. and White, P. C. (1989). Cloning and expression of rat cDNA encoding corticosteroid 11 beta-dehydrogenase. J Biol Chem **264**(32): 18939-43.

Albiston, A. L., Obeyesekere, V. R., Smith, R. E. and Krozowski, Z. S. (1994). Cloning and tissue distribution of the human 11 beta-hydroxysteroid dehydrogenase type 2 enzyme. Mol Cell Endocrinol **105**(2): R11-7.

Appelberg, R. (1994). Protective role of interferon gamma, tumor necrosis factor alpha and interleukin-6 in Mycobacterium tuberculosis and M. avium infections. Immunobiology **191**(4-5): 520-5.

Araneo, B. A., Shelby, J., Li, G. Z., Ku, W. and Daynes, R. A. (1993). Administration of dehydroepiandrosterone to burned mice preserves normal immunologic competence. Arch Surg **128**: 318-325.

Ayroldi, E., Cannarile, L., Migliorati, G., Bartoli, A., Nicoletti, I. and Riccardi, C. (1995). CD44 (Pgp-1) inhibits CD3 and dexamethasone-induced apoptosis. Blood **86**: 2672-8.

Baker, R., Zumla, A. and Rook, G. A. W. (1996). Tuberculosis, steroid metabolism and immunity. Q J Med **89**(5): 387-394.

Baker, R. W., Walker, B. R., Shaw, R. J., Honour, J., Jessop, D. S., Lightman, S. L., Rook, G. A. W. and Zumla, A. (2000). Increased cortisol: cortisone ratio in acute pulmonary tuberculosis. Am J Resp Crit Care Med **In press**.

Barnes, D. J., Naraqi, S., Temu, P. and Turtle, J. R. (1989). Adrenal function in patients with active tuberculosis. Thorax **44**: 422-424.

Barnes, P. F., Lu, S., Abrams, J. S., Wang, E., Yamamura, M. and Modlin, R. L. (1993). Cytokine production at the site of disease in tuberculosis. Infect Immun **61**: 3482-3489.

Barr, R. G. and Menzies, R. (1994). The effect of war on tuberculosis. Results of a tuberculin survey among displaced persons in El Salvador and a review of the literature. Tuber Lung Dis **75**(4): 251-9.

Batuman, O. A., Ferrero, A., Cupp, C., Jimenez, S. A. and Khalili, K. (1995). Differential regulation of transforming growth factor beta-1 gene expression by glucocorticoids in human T and glial cells. J Immunol **155**(9).

Batuman, O. A., Ferrero, A. P., Diaz, A. and Jimenez, S. A. (1991). Regulation of transforming growth factor-beta 1 gene expression by glucocorticoids in normal human T lymphocytes. J Clin Invest **88**: 1574-1580.

Berliner, D. L. and Dougherty, T. F. (1961). Hepatic and extrahepatic regulation of corticosteroids. Pharmacol Rev **13**: 329-359.

Bernton, E., Hoover, D., Galloway, R. and Popp, K. (1995). Adaptation to chronic stress in military trainees. Adrenal androgens, testosterone, glucocorticoids, IGF-1 and immune fucntion. Ann NY Acad Sci **774**: 217-231.

Besedovsky, H. O., del-Rey, A., Klusman, I., Furukawa, H., Monge-Arditi, G. and Kabiersch, A. (1991). Cytokines as modulators of the hypothalamus-pituitary-adrenal axis. J Steroid Biochem Mol Biol **40**: 613-618.

Best, R., Nelson, S. M. and Walker, B. R. R. (1997). Dexamethasone and 11 dehydroxydexamethasone as tools to investigate the isozymes of 11 β–hydroxysteroid dehydrogenase in vitro and in vivo. J Endocrinol **153**: 41-48.

Blauer, K. L., Poth, M., Rogers, W. M. and Bernton, E. W. (1991). Dehydroepiandrosterone antagonises the suppressive effects of dexamethasone on lymphocyte proliferation. Endocrinology **129**(6): 3174-3179.

Bluthe, R. M., Michaud, B., Kelley, K. W. and Dantzer, R. (1996). Vagotomy blocks behavioural effects of interleukin-1 injected via the intraperitoneal route but not via other systemic routes. Neuroreport **7**(15-17): 2823-7.

Bohle, B., Willheim, M., Baier, K., Stadler, B., Spitzauer, S., Scheiner, O., Kraft, D. and Ebner, C. (1995). Hydrocortisone enhances total IgE levels--but not the synthesis of allergen-specific IgE--in a monocyte-dependent manner. Clin Exp Immunol **101**: 474-479.

Bray, P. J., Du, B., Mejia, V. M., Hao, S. C., Deutsch, E., Fu, C., Wilson, R. C., Hanauske-Abel, H. and McCaffrey, T. A. (1999). Glucocorticoid resistance caused by reduced expression of the glucocorticoid receptor in cells from human vascular lesions. Arterioscler Thromb Vasc Biol **19**(5): 1180-9.

Brem, A. S., Bina, R. B., King, T. C. and Morris, D. J. (1998). Localisation of 2 11beta-hydroxysteroid dehydrogenase isoforms in aortic endothelial cells. Hypertension **31**(2): 459-62.

Brinkman, V. and Kristofic, C. (1995). Regulation by corticosteroids of Th1 and Th2 cytokine production in human CD4+ effector T cells generated from CD45RO- and CD45RO+ subsets. J Immunol(155): 3322-8.

Brown, D. H., LaFuse, W. and Zwilling, B. S. (1995). Cytokine-mediated activation of macrophages from Mycobacterium bovis BCG-resistant and -susceptible mice: differential effects of corticosterone on antimycobacterial activity and expression of the Bcg gene (candidate Nramp). Infect Immun **63**: 2983-2988.

Brown, D. H., Miles, B. A. and Zwilling, B. S. (1995). Growth of Mycobacterium tuberculosis in BCG-resistant and -susceptible mice: establishment of latency and reactivation. Infect Immun **63**: 2243-2247.

Brown, D. H., Sheridan, J., Pearl, D. and Zwilling, B. S. (1993). Regulation of mycobacterial growth by the hypothalamus-pituitary-adrenal axis: Differential responses of Mycobacterium bovis BCG-resistant and -susceptible mice. Infect Immun **61**: 4793-4800.

Brunetti, M., Martelli, N., Colasante, A., Piantelli, M., Musiani, P. and Aiello, F. B. (1995). Spontaneous and glucocorticoid-induced apoptosis in human mature T lymphocytes. Blood **86**: 4199-205.

Bujalska, I. J., Kumar, S. and Stewart, P. M. (1997). Does central obesity reflect "Cushing's disease of the omentum"? Lancet **349**(9060): 1210-3.

Cherwinski, H. M., Schumacher, J. H., Brown, K. D. and Mosmann, T. R. (1987). Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J Exp Med **166**(5): 1229-44.

Chowdrey, H. S., Larsen, P. J., Harbuz, M. S., Jessop, D. S., Aguilera, G., Eckland, D. J. and Lightman, S. L. (1995). Evidence for arginine vasopressin as the primary activator of the HPA axis during adjuvant-induced arthritis. Br J Pharmacol **116**(5): 2417-24.

Cooper, A. M., Roberts, A. D., Rhoades, E. R., Callahan, J. E., Getzy, D. M. and Orme, I. M. (1995). The role of interleukin-12 in acquired immunity to Mycobacterium tuberculosis infection. Immunology **84**: 423-432.

Cooper, R. C., Bouic, P. J. D., Beyers, N., Gie, R. P. and Donald, P. R. (1995). The measurement of serum cytokines in adolescents with pulmonary tuberculosis. Glaxo Action TB Sci Conf & Workshops, 5 6 October **Abstract**.

Corrigan, C. J., Hamid, Q., North, J., Barkans, J., Moqbel, R., Durham, S., Gemou, E. V. and Kay, A. B. (1995). Peripheral blood CD4 but not CD8 T-lymphocytes in patients with exacerbation of asthma transcribe and translate messenger RNA encoding cytokines which prolong eosinophil survival in the context of a Th2-type pattern: effect of glucocorticoid therapy. Am J Respir Cell Mol Biol **12**: 567-78.

Crowley, S., Hindmarsh, P. C., Brook, C. G. D. and Honour, J. W. (1993). Reproducibility and repeatability of low dose ACTH stimulation tests. J Endocrinol **136**: 167-172.

Crowley, S., Holownia, P., Hindmarsh, P., Brook, C. G. D. and Honour, J. W. (1991). The acute adrenal response to physiological ACTH. J Endocrinol **130**: 475-479.

Cunnah, D., Jessop, D. S., Besser, G. M. and Rees, L. H. (1987). Measurement of circulating corticotrophin-releasing factor in man. J Endocrinol **113**(1): 123-31.

Danenberg, H. D., Ben-Yehuda, A., Zakay-Rones, Z. and Friedman, G. (1995). Dehydroepiandrosterone enhances influenza vaccination in aged mice. Ann NY Acad Sci **774**: 297-299.

Daynes, R. A. and Araneo, B. A. (1989). Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factos IL-2 and IL-4. Eur J Immunol **19**: 2319-2325.

Daynes, R. A., Araneo, B. A., Dowell, T. A., Huang, K. and Dudley, D. (1990). Regulation of murine lymphokine production in vivo. III. The lymphoid tissue microenvironment exerts regulatory influences over T helper cell function. J Exp Med **171**: 979-996.

Daynes, R. A., Araneo, B. A., Ershler, W. B., Maloney, C., Li, G.-Z. and Ryu, S.-Y. (1993). Altered regulation of IL-6 production with normal aging; possible linkage to the age-associated decline in dehydroepiandrosterone and its sulphated derivative. J Immunol **150**: 5219-5230.

Daynes, R. A., Araneo, B. A., Hennebold, J., Enioutina, J. and Mu, H. H. (1995). Steroids as regulators of the mammalian immune response. J Invest Dermatol **105**: 14S-19S.

Daynes, R. A., Dowell, T. and Araneo, B. A. (1991). Platelet-derived growth factor is a potent biologic response modifier of T cells. J Exp Med **174**: 1323-1333.

Deckx, R. and De Moor, P. (1996). Study of 11-beta hydroxysteroid dehydrogenase in vitro. 1. Biochemical characterisation in spleen homogenate. Pflugers Archive **289**: 59.

Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J. and Wahli, W. (1996). The PPARα-leukotriene B4 pathway to inflammation control. Nature **384**: 39-43.

Dougherty, T. F., Berliner, M. L. and Berliner, D. L. (1960). 11β-hydroxy dehydrogenase system activity in thymi of mice following prolonged cortisol treatment. Endocrinology **66**: 550-558.

Edwards, C. R. W., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., de, K. E. and Monder, C. (1988). Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. Lancet **2**(8618): 986-9.

Elliott, L. H., Levay, A. K., Sparks, B., Miller, M. and Roszman, T. L. (1996). Dexamethasone and prostaglandin E2 modulate T-cell receptor signaling through a cAMP-independent mechanism. Cell Immunol **169**(1): 117-24.

Ellis, M. E. and Tayoub, F. (1986). Adrenal function in tuberculosis. Br J Dis Chest **80**: 7-12.

Ellner, J. J. (1991). Regulation of the human cellular immune response to Mycobacterium tuberculosis. The mechanism of selective depression of the response to PPD. Bull Int Union Tuberc Lung Dis **66**: 129-32.

Ellner, J. J. (1997). Regulation of the human immune response during tuberculosis. J Lab Clin Med **130**(5).

Erard, F., Nabholz, M., Dupuy-D'Angeac, A. and MacDonald, H. R. (1985). Differential requirements for the induction of interleukin 2 responsiveness in L3T4+ and Lyt-2+ T cell subsets. J Exp Med **162**(5): 1738-43.

Escher, G., Frey, F. J. and Frey, B. M. (1994). 11 beta-Hydroxysteroid dehydrogenase accounts for low prednisolone/prednisone ratios in the kidney. Endocrinology **135**(1): 101-6.

Escher, G., Galli, I., Vishwanath, B. S., Frey, B. M. and Frey, F. J. (1997). Tumor necrosis factor alpha and interleukin 1beta enhance the cortisone/cortisol shuttle. J Exp Med **186**(2): 189-98.

Evangelatou, M., Antoniw, J. and Cooke, B. (1996). The effect of leukocytes on 11β - HSD activity in human granulosar cell cultures. J Endocrinol(148): Abstract p.55.

Few, J. D., Chaudry, S. and James, V. H. D. (1984). The direct determination of aldosterone in human saliva. J Steroid Biochem **21**: 87-92.

Fischer, A. and Konig, W. (1991). Influence of cytokines and cellular interactions on the glucocorticoid-induced Ig (E, G, A, M) synthesis of peripheral blood mononuclear cells. Immunology **74**: 228-233.

Friedland, J. S., Hartley, J. C., Hartley, C. G., Shattock, R. J. and Griffin, G. E. (1995). Inhibition of ex vivo proinflammatory cytokine secretion in fatal Mycobacterium tuberculosis infection. Clin Exp Immunol **100**(2): 233-8.

Friedland, J. S., Shattock, R. J., Johnson, J. D., Remick, D. G., Holliman, R. E. and Griffin, G. E. (1993). Differential cytokine gene expression and secretion after phagocytosis by a human monocytic cell line of Toxoplasma gondii compared with Mycobacterium tuberculosis. Clin Exp Immunol **91**(2): 282-6.

Fukuda, R., Ichikawa, Y., Takaya, M., Ogawa, Y. and Masumoto, A. (1994). Circadian variations and prednisolone-induced alterations of circulating lymphocyte subsets in man. Intern Med **33**(12): 733-8.

Funder, J. W., Pearce, P. T., Smith, R. and Smith, A. I. (1988). Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science **242**: 583-585.

Gao, H. B., Ge, R. S., Lakshmi, V., Marandici, A. and Hardy, M. P. (1997). Hormonal regulation of oxidative and reductive activities of 11 beta- hydroxysteroid dehydrogenase in rat Leydig cells. Endocrinology **138**(1): 156-61.

Garg, M. and Bondada, S. (1993). Reversal of age-associated decline in immune response to Pnu-Imune vaccine by supplementation with the steroid hormone dehydroepiandrosterone. Infect Immun **61**: 2238-2241.

Gelding, S. V., Taylor, N. F., Wood, P. J., Noonan, K., Weaver, J. U., Wood, D. F. and Monson, J. P. (1998). The effect of growth hormone replacement therapy on cortisol-cortisone interconversion in hypopituitary adults: evidence for growth hormone modulation of extrarenal 11 beta-hydroxysteroid dehydrogenase activity. Clin Endocrinol (Oxf) **48**(2): 153-62.

Glaser, R., Lafuse, W. P., Bonneau, R. H., Atkinson, C. and Kiecolt, G. J. (1993). Stress-associated modulation of proto-oncogene expression in human peripheral blood leukocytes. Behav Neurosci **107**(3): 525-9.

Gomez-Sanchez, E. P., Cox, D., Foecking, M., Ganjam, V. and Gomez-Sanchez, C. E. (1996). 11-beta hydroxysteroid dehydrogenases of the choriocarcinoma line JEG-3 and their inhibition by glycyrrhetinic acid and other natural substances. Steroids **61**: 110.

Guida, L., O'Hehir, R. E. and Hawrylowicz, C. M. (1994). Synergy between dexamethasone and interleukin-5 for the induction of major histocompatibility complex class II expression by human peripheral blood eosinophils. Blood **84**: 2733-2740.

Haanaes, O. C. and Bergmann, A. (1983). Tuberculosis emerging in patients treated with corticosteroids. Eur J Respir Dis **64**(4): 294-7.

Harbuz, M. S., Rees, R. G., Eckland, D., Jessop, D. S., Brewerton, D. and Lightman, S. L. (1992). Paradoxical responses of hypothalamic CRF mRNA and CRF-41 peptide and adenohypophyseal POMC mRNA during chronic inflammatory stress. Endocrinology **130**: 1394-1400.

Hennebold, J. D. (1997). Active catabolism of glucocorticoids by 11b-HSD in vivo is a necessary requirement for natural resistance to infection with Listeria monocytogenes. Int Immunol **9**: 105-115.

Hennebold, J. D., Ryu, S. Y., Mu, H. H., Galbraith, A. and Daynes, R. A. (1996). 11 beta-hydroxysteroid dehydrogenase modulation of glucocorticoid activities in lymphoid organs. Am J Physiol **270**(6 Pt 2): R1296-306.

Hernandez Pando, R., Orozco, H., Arriaga, K., Sampieri, A., Larriva Sahd, J. and Madrid Marina, V. (1997). Analysis of the local kinetics and localization of interleukin-1 alpha, tumour necrosis factor-alpha and transforming growth factor-beta, during the course of experimental pulmonary tuberculosis. Immunology **90**(4): 607-17.

Hernandez Pando, R., Orozco, H., Honour, J., Silva, P., Leyva, R. and Rook, G. A. (1995). Adrenal changes in murine pulmonary tuberculosis; a clue to pathogenesis? FEMS Immunol Med Microbiol **12**(1): 63-72.

Hernandez-Pando, R., De La Luz Streber, M., Orozco, H., Arriaga, K., Pavon, L., Al-Nakhli, S. A. and Rook, G. A. (1998). The effects of androstenediol and dehydroepiandrosterone on the course and cytokine profile of tuberculosis in BALB/c mice. Immunology **95**(2): 234-41.

Hernandez-Pando, R., de la Luz Streber, M., Orozco, H., Arriaga, K., Pavon, L., Marti, O., Lightman, S. L. and Rook, G. A. W. (1998). Emergent immunoregulatory properties of combined glucocorticoid and anti-glucocorticoid steroids in a model of tuberculosis. Q J Med **91**: 755-66.

Hernandez-Pando, R., Orozcoe, H., Sampieri, A., Pavon, L., Velasquillo, C., Larriva-Sahd, J., Alcocer, J. M. and Madrid, M. V. (1996). Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. Immunology **89**(1): 26-33.

Hernandez-Pando, R., Pavon, L., Arriaga, K., Orozco, H., Madrid-Marina, V. and Rook, G. (1997). Pathogenesis of tuberculosis in mice exposed to low and high doses of an environmental mycobacterial saprophyte before infection. Infect Immun **65**(8): 3317-3327.

Hiemke, C., Brunner, R., Hammes, E., Muller, H., Meyer, z. B. K. and Lohse, A. W. (1995). Circadian variations in antigen-specific proliferation of human T lymphocytes and correlation to cortisol production. Psychoneuroendocrinology **20**(3): 335-42.

Hill, A. D., Naama, H. A., Gallagher, H. J., Shou, J., Calvano, S. E. and Daly, J. M. (1995). Glucocorticoids mediate macrophage dysfunction in protein calorie malnutrition. Surgery **118**: 130-16;.

Homma, M., Onodera, T., Hirabatake, M., Oka, K., Kanazawa, M., Miwa, T. and Hayashi, T. (1998). Activation of 11 beta-hydroxysteroid dehydrogenase by dehydroepiandrosterone sulphate as an anti-hypertensive agent in spontaneously hypertensive rats. J Pharm Pharmacol **50**(10): 1139-45.

Hubbard, W. C., Bickel, C. and Schleimer, R. P. (1994). Simultaneous quantitation of endogenous levels of cortisone and cortisol in human nasal and bronchoalveolar lavage fluids and plasma via gas chromatography-negative ion chemical ionization mass spectrometry. Anal Biochem **221**: 109-117.

Jamieson, P. M., Chapman, K. E., Edwards, C. R. W. and Seckl, J. R. (1995). 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta- reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. Endocrinology **136**(11): 4754-61.

Jesse, R. L., Loesser, K., Eich, D. M., Qian, Y. Z., Hess, M. L. and Nestler, J. E. (1995). Dehydroepiandrosterone inhibits human platelet aggregation in vitro and in vivo. Ann NY Acad Sci **774**: 281-290.

Jones, T. H. and Kennedy, R. L. (1993). Cytokines and hypothalamic-pituitary function. Cytokine **5**(6): 531-8.

Joyce, D. A., Steer, J. H. and Abraham, L. J. (1997). Glucocorticoid modulation of human monocyte/macrophage function: control of TNF-alpha secretion. Inflamm Res **46**(11): 447-51.

Jung, T., Schauer, U., Heusser, C., Neumann, C. and Rieger, C. (1993). Detection of intracellular cytokines by flow cytometry. J Immunol Methods **159**: 197-207.

King, L. B. and Ashwell, J. D. (1993). Signaling for death of lymphoid cells. Curr Opin Immunol **5**(3): 368-73.

Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T. and Kanakubo, Y. (1987). P-450 HFLa, a form of cytochrome P-450 purified from human foetal livers, is the 16α-hydroxylase of dehydroepiandrosterone 3-sulphate. J Biol Chem **262**: 13534-13537.

Klein, A., Lishner, M., Bruser, B., Curtis, J. E., Amato, D. J. and Malkin, A. (1990). Cortisol catabolism by lymphocytes of patients with chronic lymphocytic leukemia. Biochem Cell Biol **68**: 810-813.

Kotelevtsev, Y., Holmes, M. C., Burchell, A., Houston, P. M., Schmoll, D., Jamieson, P., Best, R., Brown, R., Edwards, C. R., Seckl, J. R. and Mullins, J. J. (1997). 11beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. Proc Natl Acad Sci USA **94**(26): 14924-9.

Kotelevtsev, Y. V., Brown, R. W., Fleming, S., Edwards, C. R. W., Seckl, J. R. and Mullins, J. J. (1999). Hypertension in mice caused lacking 11b-hydroxysteroid dehydrogenase type 2. J Clin Invest **103**(5): 683-8.

Kovacs, K. J. and Elenkov, I. J. (1995). Differential dependence of ACTH secretion induced by various cytokines on the integrity of the paraventricular nucleus. J Neuroendocrinol **7**(1): 15-23.

Krouwels, F. H., van der Heijden, J. F., Lutter, R., van Neerwen, R. J. J., Jansen, H. M. and Out, T. A. (1996). Glucocorticoids affect functions of airway- and blood-derived human T cell clones, favoring the Th1 profile through two mechanisms. Am J Respir Cell Mol Biol **14**: 388-397.

Law, K., Weiden, M., Harkin, T., Tchou Wong, K., Chi, C. and Rom, W. N. (1996). Increased release of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha by bronchoalveolar cells lavaged from involved sites in pulmonary tuberculosis. Am J Respir Crit Care Med **153**(2): 799-804.

Laye, S., Bluthe, R. M., Kent, S., Combe, C., Medina, C., Parnet, P., Kelley, K. and Dantzer, R. (1995). Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS. Am J Physiol **268**: R1327-1331.

Lemmer, B., Schwulera, U., Thrun, A. and Lissner, R. (1992). Circadian rhythm of soluble interleukin-2 receptor in healthy individuals. Eur Cytokine Netw **3**(3): 335-6.

Lortholary, O., Christeff, N., Casassus, P., Thobie, N., Veyssier, P., Trogoff, B., Torri, O., Brauner, M., Nunez, E. A. and Guillevin, L. (1996). Hypothalamo-pituitary-adrenal function in human immunodeficiency virus-infected men. J Clin Endocrinol Metab **81**(2): 791-6.

Malone, J. L., Simms, T. E., Gray, G. C., Wagner, K. F., Burge, J. R. and Burke, D. S. (1990). Sources of variability in repeated T-helper lymphocyte counts from human immunodeficiency virus type 1-infected patients: total lymphocyte count fluctuations and diurnal cycle are important. J Acquir Immune Defic Syndr **3**(2): 144-51.

Martini, E., Muller, J. Y., Doinel, C., Gastal, C., Roquin, H., Douay, L. and Salmon, C. (1988). Disappearance of CD4-lymphocyte circadian cycles in HIV-infected patients: early event during asymptomatic infection. Aids **2**(2): 133-4.

McAllister, W. A., Thompson, P. J., Al-Habet, S. M. and Rogers, H. J. (1983). Rifampicin reduces effectiveness and bioavailability of prednisolone. Br Med J ClinRes Ed **286**(6369): 923-5.

McConkey, D. J., Jondal, M. B. and Orrenium, S. G. (1994). Chemical-induced apoptosis in the immune system. Immunotoxicology and Immunopharmacology. J. H. Dean, M. I. Luster, A. E. Munson and I. Kimber. New York, Raven Press: 473-485.

Meagher, L. C., Cousin, J. M., Seckl, J. R. and Haslett, C. (1996). Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol **156**: 4422-4428.

Meikle, A. W., Dorchuck, R. W., Araneo, B. A., Stringham, J. D., Evans, T. G., Spruance, S. L. and Daynes, R. A. (1992). The presence of a dehydroepiandrosterone-specific receptor-binding complex in murine T cells. J Steroid Biochem Molec Biol **42**: 293-304.

Menard, J. and Catt, K. J. (1972). Measurement of renin activity , concentration and substrate in rat plasma by radioimmunoassay of angiotensin I. Endocrinology **90**: 422-30.

Migliorati, G., Pagliacci, C., Moraca, R., Crocicchio, F., Nicoletti, I. and Riccardi, C. (1992). Interleukins modulate glucocorticoid-induced thymocyte apoptosis. Int J Clin Lab Res **21**(4): 300-3.

Modlin, R. L. (1994). Th1-Th2 paradigm: insights from leprosy. J Invest Dermatol **102**(6): 828-32.

Morales, A. J., Nolan, J., Nelson, J. and Yen, A. S. (1994). Effects of replacement dose of DHEA in men and women of advancing age. J Clin Endocrinol Metab **78**: 1360-1367.

Morfin, R. and Courchay, G. (1994). Pregnenolone and dehydroepiandrosterone as precursors of native 7-hydroxylated metabolites which increase the immune response in mice. J Steroid Biochem Molec Biol **50**: 91-100.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986). Two types of murine helper T cell clone. 1) Definition according to profiles of lymphokine activities and secreted proteins. J Immunol **136**: 2348-2357.

Newport, M. J., Huxley, C. M., Huston, S., Hawrylowicz, C. M., Oostra, B. A., Williamson, R. and Levin, M. (1996). A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N Engl J Med **335**(26): 1941-9.

Nicod, L. P., Lipscomb, M. F., Weissler, J. C., Lyons, C. R., Albertson, J. and Toews, G. B. (1987). Mononuclear cells in human lung parenchyma. Characterization of a potent accessory cell not obtained by bronchoalveolar lavage. Am Rev Respir Dis **136**(4): 818-23.

Nimmagadda, S. R., Szefler, S. J., Spahn, J. D., Surs, W. and Leung, D. Y. (1997). Allergen exposure decreases glucocorticoid receptor binding affinity and steroid responsiveness in atopic asthmatics. Am J Respir Crit Care Med **155**(1): 87-93.

Northrop, J. P., Crabtree, G. R. and Mattila, P. S. (1992). Negative regulation of interleukin-2 transcription by the glucocorticoid receptor. J Exp Med **175**: 1235-1241.

Oakley, R. H., Sar, M. and Cidlowski, J. A. (1996). The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. J Biol Chem **271**(16): 9550-9.

Olsen, N. J., Zhou, P. and Ong, H. (1993). Testosterone induces expression of transforming growth factor-β in the murine thumus. Steroid Biochem Mol Biol **45**: 327-332.

Onwubalili, J. K., Scott, G. M. and Smith, H. (1986). Acute respiratory distress related to chemotherapy of advanced pulmonary tuberculosis: a study of two cases and review of the literature. QJM **59**: 599-610.

Opstad, P. K. (1992). Androgenic hormones during prolonged physical stress, sleep and energy deficiency. J Clin Endocrinol Metab **74**: 1176-1183.

Opstad, P. K. (1994). Circadian rhythm of hormones is extinguished during prolonged physical stress, sleep and energy deficiency in young men. Eur J Endocrinol **131**: 56-66.

Orphanidou, D., Gaga, M., Rasidakis, A., Dimakou, K., Toumbis, M., Latsi, P., Pandalos, J., Christacopoulou, J. and Jordanoglou, J. (1996). Tumour necrosis factor, interleukin-1 and adenosine deaminase in tuberculous pleural effusion. Respir Med **90**(2): 95-8.

Padgett, D. A. and Loria, R. M. (1994). In vitro potentiation of lymphocyte activation by dehydroepiandrosterone, androstenediol and androstenetriol. J Immunol **153**: 1544-1552.

Padgett, D. A., Sheridan, J. F. and Loria, R. (1995). Steroid hormone regulation of a polyclonal Th2 immune response. Ann NY Acad Sci **774**: 323-325.

Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Sampognaro, S., Piccinni, M. P., Macchia, D., Maggi, E., Del Prete, G. and Romagnani, S. (1992). IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. J Immunol **149**(9): 2977-83.

Pechhold, K., Wesch, D., Schondelmaier, S. and Kabelitz, D. (1994). Primary activation of V gamma 9-expressing gamma delta T cells by Mycobacterium tuberculosis. Requirement for Th1-type CD4 T cell help and inhibition by IL-10. J Immunol **152**(10): 4984-92.

Perlstein, R. S., Mougey, E. H., Jackson, W. E. and Neta, R. (1991). Interleukin-1 and interleukin-6 act synergistically to stimulate the release of adrenocorticotropic hormone in vivo. Lymphokine Cytokine Res **10**(1-2): 141-6.

Peters, J. M., Yuan-Chun, Z., Ram, P. A., Lee, S. S. T., Gonzalez, F. J. and Waxman, D. J. (1996). Peroxisome proliferator-activated receptor α required for gene induction by dehydroepiandrosterone-3β-sulphate. Mol Pharmacol **50**: 67-74.

Peterson, J. D., Waltenbaugh, C. and Miller, S. D. (1992). IgG subclass responses to Theiler's murine encephalomyelitis virus infection and immunization suggest a dominant role for Th1 cells in susceptible mouse strains. Immunology **75**: 652-658.

Petrovsky, N., McNair, P. and Harrison, L. C. (1994). Circadian rhythmicity of interferon-gamma production in antigen-stimulated whole blood. Chronobiologia **21**(3-4): 293-300.

Ploem, J. and Tanke, H. (1987). Introduction to Fluorescence Microscopy. Oxford, Oxford University Press.

Polak, J. and Van Noorden, S. (1984). An introduction to Immunocytochemistry: Current techniques and problems. Oxford, Oxford University Press.

Post, F. A., Soule, S. G., Willcox, P. A. and Levitt, N. S. (1994). The spectrum of endocrine dysfunction in active pulmonary tuberculosis. Clin Endocrinol **40**: 367-371.

Pownall, R., Kabler, P. A. and Knapp, M. S. (1979). The time of day of antigen encounter influences the magnitude of the immune response. Clin Exp Immunol **36**(2): 347-54.

Rajan, V., Chapman, K. E., Lyons, V., Jamieson, P., Mullins, J. J., Edwards, C. R. W. and Seckl, J. R. (1995). Cloning, sequencing and tissue-distribution of mouse 11 beta-hydroxysteroid dehydrogenase-1 cDNA. J Steroid Biochem Mol Biol **52**(2): 141-7.

Ramirez, F., Fowell, D. J., Puklavec, M., Simmonds, S. and Mason, D. (1996). Glucocorticoids promote a Th2 cytokine response by CD4+ T cells in vitro. J Immunol **156**(7): 2406-12.

Rasmussen, K. R., Martin, E. G. and Healey, M. C. (1993). Effects of dehydroepiandrosterone in immunosuppressed rats infected with Cryptosporidium parvum. J Parasitol **79**: 364-370.

Reznek, R. H. and Armstrong, P. (1994). The adrenal gland. Clin Endocrinol **40**: 561-576.

Robel, P. and Baulieu, E. E. (1995). Dehydroepiandrosterone (DHEA) is a neuroactive neurosteroid. Ann NY Acad Sci **774**: 82-110.

Roberts, N. A., Barton, R. N. and Horan, M. A. (1990). Ageing and sensitivity of the adrenal gland to physiological doses of ACTH in man. J Endocrinol **126**: 507-513.

Robinson, D. S., Ying, S., Taylor, I. K., Wangoo, A., Mitchell, D. M., Kay, A. B., Hamid, Q. and Shaw, R. J. (1994). Evidence for a Th1-like bronchoalveolar T-cell subset and predominance of interferon-gamma gene activation in pulmonary tuberculosis. Am J Respir Crit Care Med **149**(4 Pt 1): 989-93.

Rook, G. and Baker, R. (1999). Cortisol metabolism, cortisol sensitivity and the pathogenesis of leprosy reactions. Trop Med Int Health **4**: 493-8.

Rook, G., Baker, R., Walker, B., Honour, J., Jessop, D., Hernandez-Pando, R., Arriaga, K., Shaw, R., Zumla, A. and Lighman, S. (2000). Local regulation of glucocorticoid activity in sites of inflammation; insights from the study of tuberculosis. Ann NY Acad Sci **In press.**

Rook, G. A. W. and Hernandez-Pando, R. (1996). The pathogenesis of tuberculosis. Ann Rev Microbiol **50**: 259-84.

Rook, G. A. W., Honour, J., Kon, O. M., Wilkinson, R. J., Davidson, R. and Shaw, R. J. (1996). Urinary steroid metabolites in tuberculosis; a new clue to pathogenesis. QJMed **89**(5): 387-394.

Rook, G. A. W., Steele, J., Ainsworth, M. and Leveton, C. (1987). A direct effect of glucocorticoid hormones on the ability of human and murine macrophages to control the growth of M. tuberculosis. Eur J Respir Dis **71**: 286-291.

Sahn, S. A. and Lakshminarayan, S. (1976). Tuberculosis after corticosteroid therapy. Br J Dis Chest **70**(3): 195-205.

Sanchez, F. O., Rodriguez, J. I., Agudelo, G. and Garcia, L. F. (1994). Immune responsiveness and lymphokine production in patients with tuberculosis and healthy controls. Infect Immun **62**(12): 5673-8.

Sarma, G. R., Chandra, I., Ramachandran, G., Krishnamurthy, P. V., Kumaraswami, V. and Prabhakar, R. (1990). Adrenocortical function in patients with pulmonary tuberculosis. Tubercle **71**: 277-282.

Saunders, R., Robson, S., Soule, S. and Kirsch, R. E. (1993). Secretion of interleukin-6 by peripheral blood mononuclear cells from patients with pulmonary tuberculosis and their contacts. J Clin Lab Immunol **40**(1): 19-28.

Schauf, V., Rom, W. N., Smith, K. A., Sampaio, E. P., Meyn, P. A., Tramontana, J. M., Cohn, Z. A. and Kaplan, G. (1993). Cytokine gene activation and modified responsiveness to interleukin-2 in the blood of tuberculosis patients. J Infect Dis **168**: 1056-1059.

Schleimer, R. P. (1991). Potential regulation of inflammation in the lung by local metabolism of hydrocortisone. Am J Respir Cell Mol Biol **4**(2): 166-73.

Scott, G. M., Murphy, P. G. and Gemidjioglu, M. E. (1990). Predicting deterioration of treated tuberculosis by corticosteroid reserve and C-reactive protein. J Infect **21**: 61-69.

Seah, G. T., Scott, G. M. and Rook, G. A. (2000). Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. J Infect Dis **181**(1): 385-9.

Sothern, R. B., Roitman-Johnson, B., Kanabrocki, E. L., Yager, J. G., Fuerstenberg, R. K., Weatherbee, J. A., Young, M. R., Nemchausky, B. M. and Scheving, L. E. (1995). Circadian characteristics of interleukin-6 in blood and urine of clinically healthy men. In Vivo **9**: 331-339.

Spath Schwalbe, E., Born, J., Schrezenmeier, H., Bornstein, S. R., Stromeyer, P., Drechsler, S., Fehm, H. L. and Porzsolt, F. (1994). Interleukin-6 stimulates the hypothalamus-pituitary-adrenocortical axis in man. J Clin Endocrinol Metab **79**(4): 1212-4.

Spencer, N. F. L., Poynter, M. E., Henebold, J. D., Mu, H. H. and Daynes, R. A. (1995). Does DHEAS restore immune competence in aged animals through its capacity to function as a natural modulator of peroxisome activities ? Ann NY Acad Sci **774**: 201-216.

Stewart, P. M., Corrie, J. E., Shackleton, C. H. and Edwards, C. R. W. (1988). Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. J Clin Invest **82**(1): 340-9.

Stewart, P. M., Wallace, A. M., Atherden, S. M., Shearing, C. H. and Edwards, C. R. W. (1990). Mineralocorticoid activity of carbenoxolone: contrasting effects of carbenoxolone and liquorice on 11 β-hydroxysteroid dehydrogenase activity in man. Clin Sci **78**: 49-54.

Stöcklin, E., Vissler, M., Gouilleux, F. and Groner, B. (1996). Functional interactions between Stat5 and the glucocorticoid receptor. Nature **383**: 726-728.

Suzuki, T., Sasano, H., Suzuki, S., Hirasawa, G., Takeyama, J., Muramatsu, Y., Date, F., Nagura, H. and Krozowski, Z. S. (1998). 11Beta-hydroxysteroid dehydrogenase type 2 in human lung: possible regulator of mineralocorticoid action. J Clin Endocrinol Metab **83**(11): 4022-5.

Suzuki, T., Suzuki, N., Daynes, R. A. and Engleman, E. G. (1991). Dehydroepiandrosterone enhances IL2 production and cytotoxic effector function of human T cells. Clin Immunol Immunopathol **61**: 202-211.

Tabardel, Y., Duchateau, J., Schmartz, D., Marecaux, G., Shahla, M., Barvais, L., Leclerc, J. L. and Vincent, J. L. (1996). Corticosteroids increase blood interleukin-10 levels during cardiopulmonary bypass in men. Surgery **119**: 76-80.

Tetsuka, M., Thomas, F. J., Thomas, M. J., Anderson, R. A., Mason, J. I. and Hillier, S. G. (1997). Differential expression of messenger ribonucleic acids encoding 11beta-hydroxysteroid dehydrogenase types 1 and 2 in human granulosa cells. J Clin Endocrinol Metab **82**(6): 2006-9.

Tobach, E. and Bloch, H. (1956). Effect of stress by crowding prior to and following tuberculous infection. Am J Physiol **187**: 399-402.

Tocque, K., Regan, M., Remmington, T., Beeching, N. J., Jamieson, I., Syed, Q. and Davies, P. D. (1999). Social factors associated with increases in tuberculosis notifications. Eur Respir J **13**(3): 541-5.

Toossi, Z. and Ellner, J. J. (1998). The role of TGF beta in the pathogenesis of human tuberculosis. Clin Immunol Immunopathol(87): 107-14.

Vacchio, M. S., Papdopoulos, V. and Ashwell, J. D. (1994). Steroid production in the thymus: implications for thymocyte selection. J Exp Med **179**: 1835-1846.

Vanham, G., Toossi, Z., Hirsch, C. S., Wallis, R. S., Schwander, S. K., Rich, E. A. and Ellner, J. J. (1997). Examining a paradox in the pathogenesis of human pulmonary tuberculosis: immune activation and suppression/anergy. Tuber Lung Dis **78**(3-4): 145-58.

Van-Wauwe, J., Aerts, F., Walter, H. and de-Boer, M. (1995). Cytokine production by phytohemagglutinin-stimulated human blood cells: effects of corticosteroids, T cell immunosuppressants and phosphodiesterase IV inhibitors. Inflamm Res **44**: 400-405.

Vieira, P. L., Kalinski, P., Wierenga, E. A., Kapsenberg, M. L. and de Jong, E. (1998). Glucocorticoids inhibit bioactive IL-12p70 production by in vitro- generated human dendritic cells without affecting their T cell stimulatory potential. J Immunol **161**: 5245-5251.

Visser, J., van Boxel, Dezaire, A., Methorst, D., Brunt, T., de Kloet, E. and Nagelkerken, L. (1998). Differential regulation of interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. Blood(**91**): 4255-64.

Waddell, B. J., Benediktsson, R. and Seckl, J. R. (1996). 11beta-Hydroxysteroid dehydrogenase type 2 in the rat corpus luteum: induction of messenger ribonucleic acid expression and bioactivity coincident with luteal regression. Endocrinology **137**(12): 5386-91.

Walker, B. R. (1994). Organ-specific actions of 11 beta-hydroxysteroid dehydrogenase in humans: implications for the pathophysiology of hypertension. Steroids **5**(2): 84-9.

Walker, B. R., Campbell, J. C., Fraser, R., Stewart, P. M. and Edwards, C. R. W. (1992). Mineralocorticoid excess and inhibition of 11 beta-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. Clin Endocrinol **37**(6): 483-92.

Walker, B. R., Connacher, A. A., Lindsay, R. M., Webb, D. J. and Edwards, C. R. W. (1995). Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. J Clin Endocrinol Metab **80**(11): 3155-9.

Walker, B. R. and Edwards, C. R. W. (1994). Licorice-induced hypertension and syndromes of apparent mineralocorticoid excess. Endocrinol Metab Clin N America **23**(2): 359-77.

Walker, B. R., Williams, B. C. and Edwards, C. R. W. (1994). Regulation of 11 beta-hydroxysteroid dehydrogenase activity by the hypothalamic-pituitary-adrenal axis in the rat. J Endocrinol **141**(3): 467-72.

Ward, L. D., Howlett, G. J., Discolo, G., Yasukawa, K., Hammacher, A., Moritz, R. L. and Simpson, R. J. (1994). High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp-130. J Biol Chem **269**(37): 23286-9.

Wegmann, T. G., Lin, H., Guilbert, L. and Mosmann, T. R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is succesful pregnancy a Th2 phenomenon? Immunology Today **14**(7): 353-356.

Whitworth, J. A., Stewart, P. M., Burt, D., Atherden, S. M. and Edwards, C. R. W. (1989). The kidney is the major site of cortisone production in man. Clin Endocrinol **31**(3): 355-61.

Wiegers, G. J., Croiset, G., Reul, J. M., Holsboer, F. and de Kloet, E. R. (1993). Differential effects of corticosteroids on rat peripheral blood T-lymphocyte mitogenesis in vivo and in vitro. Am J Physiol **265**: E825-E830.

Wiegers, G. J., Reul, J. M., Holsboer, F. and de Kloet, E. R. (1994). Enhancement of rat splenic lymphocyte mitogenesis after short term preexposure to corticosteroids in vitro. Endocrinology **135**(6): 2351-7.

Wilckens, T. (1995). Glucocorticoids and immune function: physiological relevance and pathological potential of hormonal dysfunction. TIPS **16**: 193-194.

Wilder, R. L. (1995). Neuroendocrine-immune system interactions and autoimmunity. Annu Rev Immunol **13**: 307-338.

Wisniewski, T. L., Hilton, C. W., Morse, E. V. and Svec, F. (1993). The relationship of serum DHEA-S and cortisol levels to measures of immune function in human immunodeficiency virus-related illness. Am J Med Sci **305**: 79-83.

Wright, B. E., Porter, J. R., Browne, E. S. and Svec, F. (1992). Antiglucocorticoid action of dehydroepiandrosterone in young obese Zucker rats. Int J Obes **16**: 579-583.

Wu, C. Y., Sarfati, M., Heusser, C., Fournier, S., Rubio-Trujillo, M., Peleman, R. and Delespesse, G. (1991). Glucocorticoids increase the synthesis of immunoglobulin E by interleukin 4-stimulated human lymphocytes. J Clin Invest **87**: 870-877.

Yokoyama, A., Maruyama, M., Ito, M., Kohno, N., Hiwada, K. and Yano, S. (1992). Interleukin 6 activity in pleural effusion. Its diagnostic value and thrombopoietic activity. Chest **102**(4): 1055-9.

Yong, A. J., Grange, J. M., Tee, R. D., Beck, J. S., Bothamley, G. H., Kemeny, D. M. and Kardjito, T. (1989). Total and anti-mycobacterial IgE levels in serum from patients with tuberculosis and leprosy. Tubercle **70**: 273-279.

Young, M. R., Matthews, J. P., Kanabrocki, E. L., Sothern, R. B., Roitman, J. B. and Scheving, L. E. (1995). Circadian rhythmometry of serum interleukin-2, interleukin-10, tumor necrosis factor-alpha, and granulocyte-macrophage colony-stimulating factor in men. Chronobiol Int **12**(1): 19-27.

Zhang, G., Zhang, L. and Duff, G. W. (1997). A negative regulatory region containing a glucocorticosteroid response element (nGRE) in the human interleukin-1beta gene. DNA Cell Biol **16**(2): 145-52.

Zhang, M., Lin, Y., Iyer, D. V., Gong, J., Abrams, J. S. and Barnes, P. F. (1995). T-cell cytokine responses in human infection with Mycobacterium tuberculosis. Infect Immun **63**(8): 3231-4.

Zumoff, B., Fukushima, D. K. and Hellman, L. (1974). Intercomparison of four methods for measuring cortisol production. J Clin Endocrinol Metab **38**: 169-174.

Zwilling, B. S. (1992). Stress affects disease outcomes. ASM News **58**(1): 23-25.